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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, A01H 5/00, 1/02 — A2

(11) International Publication Number: **WO 99/46396**

(43) International Publication Date: 16 September 1999 (16.09.99)

(21) International Application Number: PCT/US99/05126

(22) International Filing Date: 9 March 1999 (09.03.99)

(30) Priority Data:
60/077,277 9 March 1998 (09.03.98) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GLYPHOSATE AS A GAMETOCIDE

(57) Abstract

Glyphosate is used as a highly selective gametocide on plants containing in their genome a first DNA molecule which confers constitutive glyphosate tolerance and a second DNA molecule which inhibits said glyphosate tolerance specifically in male reproductive tissue. Plants containing the first and second DNA molecules are rendered male sterile by exposure to glyphosate. The methods and compositions of the invention are advantageous for use in the generation of hybrid seed, for restricting outcrossing, and for prolonging flower life.

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GLYPHOSATE AS A GAMETOCIDE

The present invention relates generally to the field of plant genetic engineering. More particularly, it concerns transgenic plants in which a male-sterile phenotype is selectively induced by application of glyphosate herbicide. The plants of the present invention are vegetatively- and female-tolerant of glyphosate, but are male-sensitive to the herbicide. The methods and compositions of the invention provide male-sterile plants for use in the production of hybrid seed, for minimizing undesirable crop outcrossing, and for lengthening flower life.

Plant biologists have long appreciated that cross-fertilization of closely related plants can result in progeny which have desirable combinations of traits not possessed by either inbred parent. This phenomenon, known as heterosis, or hybrid vigor, has been identified in major crop species (Stuber, 1994). As plants produced from such hybrid seed can result in substantially superior agronomic performance characteristics, including plant size, grain yield, disease resistance, herbicide tolerance, climatic adaptation, and others, there has been much interest in exploiting this approach for commercially produced crops. Hybrid varieties have had a huge impact on worldwide food production and have great potential for providing high yielding crop plants for the worlds' growing population.

Hybrid seed production requires that cross-pollination predominates over self-pollination, and a number of techniques have been developed which attempt to overcome the obstacle of self-pollination. A major limitation in the production of hybrid seed for most crop species, however, is the lack of simple, reliable and economical methods of generating male-sterility while leaving female gametes intact and accessible for pollination by a suitable pollen donor.

Methods of generating male sterility can be broadly classified into physical, chemical, and/or biological approaches. In some plants, such as corn, physical removal of the organ containing male gametes is relatively straightforward since the organ is both exposed and spatially separated from the female gametes. Most crop species, however, have both functional male and female organs within the same flower so that emasculation is neither simple or straightforward. Physical methods for generating male sterility are generally very labor intensive and expensive. Furthermore, it is difficult using these approaches to

ensure the complete absence of self-pollination. Thus, the development of alternative approaches which do not require laborious manual or mechanical detassling could provide substantial cost of production improvements.

In addition to physical methods for generating male sterility, chemical gametocides can be used in hybrid seed production to impart transitory male-sterility through the inhibition of viable pollen production. An effective gametocide is a compound that when applied to a plant at an appropriate developmental stage or before sexual maturity is capable of killing or effectively terminating the development of a plant's male gametes while leaving the plant's female gametes, or at least a significant proportion of them, capable of under going cross-pollination. For an effective gametocide, it is desired that the application level at which male gametes are destroyed is significantly lower than that required to destroy the female gametes. Thus, a gametocide should be capable of being applied in the field without extraordinary precautions against accidental overdoses.

Commercial production of hybrid seed using gametocides is limited primarily by their lack of selectivity for gametes in general, and for male-gametes in particular. Many compounds are capable of destroying or impairing the male gametes of a plant; indeed almost any systemic herbicide is effective in this role. However, most of these compounds also kill the female gametes, as well as vegetative tissues of the plant. Unfortunately, compounds that do possess some selectivity in targeting the gametes to a greater extent than vegetative tissues are generally non-discriminating regarding the sex of the gametes destroyed. In addition, many chemical gametocides which have shown good selectivity have toxicological issues or other environmental issues which limit the use of these compounds for production of commercial levels of hybrid seeds. Thus, methods which could improve the selectivity and the environmental safety of gametocides would have widespread applications in the production of hybrid seed.

Several naturally occurring, genetic mechanisms of male sterility exist which have been exploited for the production of hybrid seed in some plant species. In many instances, male sterility results from the developmental arrest of the pollen and/or the anther tissue which nourish the developing pollen grains and release the mature pollen with the correct timing. Hybridization strategies using CMS systems have been successfully employed in some

plant species. A disadvantage of this approach is that it requires three distinct lines to produce a single crossed hybrid: the male-sterile line (female parent), a maintainer line which is isogenic to the male-sterile line but contains fully functional mitochondria, and the male parent line.

Many CMS types have unfavorable characteristics that restrict their use; these include a linked or pleiotropic undesirable characteristic such as disease susceptibility, breakdown of sterility, inconsistent and/or complexly inherited fertility restoration. Furthermore, CMS is unavailable in many important crop species and full sterility due to the CMS cytoplasm is not always exhibited in different nuclear genetic backgrounds within a species. In those species in which CMS is widely used in hybrid seed production, there is an unsafe dependence on a single sterile cytoplasm (Williams and Levings, 1992). The southern corn leafblight caused by *Helminthosporium maydis*, Race T, which severely attacked all maize hybrids with cytoplasmic male-sterile T cytoplasm, demonstrates the vulnerability of a hybrid seed industry which relies too heavily on a single source of a male-sterile cytoplasm.

Genetic engineering has the potential to make a significant contribution to agricultural productivity by providing economical alternatives to the methods that are currently used for producing hybrid seed (Williams, 1995). For example, selective expression of genes encoding cytotoxic proteins can allow for the production of a uniform population of male sterile plants. In one example, a cytotoxic gene, barnase, expressed by a tobacco tapetal-specific promoter in anther tapetal cells, caused male sterility which could be restored in progeny when crossed with a plant containing a tapetal-specific promoter driving the expression of the barstar gene (Marini et al., 1990; Zhan et al., 1996). This same combination of barnase/barstar genes has been used to ablate specific anther cell types useful for the identification of cell types necessary for the maturation of anthers and pollen release (Goldberg et al., 1995; Beals and Goldberg, 1997).

The expression of a DAM-methylase cytotoxic to pollen formation when expressed in anthers by an anther specific promoter has been disclosed as a method of genetically engineered male sterility (WO 9617945).

Antisense RNA strategies have also been attempted for generating male sterile plants. The expression of RNA complementary to an endogenous gene critical for proper growth and development of the anthers or pollen has been suggested, such as by inhibiting expression of an essential amino acid by antisense to an aspartokinase in pollen or tapetal cells (EP 93109226), or the QM gene in maize (U.S. Patent No. 5,583,210). Fabijanski and Arnison (U.S. Patent No. 5,356,799) suggest an antisense RNA strategy involving the use of antibiotic or herbicide resistance genes, but failed to demonstrate successful use of such an approach for producing male sterile plants. Also the expression of metabolically active enzymes such as an ATPase (Zabaleta et al., 1996) in pollen or associated cell have been reported to result in male sterility. Unfortunately, many of these methods have limited utility since crossing is required to restore fertility and seed production from breeding lines can be problematic.

Outcrossing refers to the distribution of genetic information by the spread of pollen to related plants. For genetically modified plants, this is often viewed as undesirable. The outcrossing of transgenic plants to related wild plant species has raised concerns about the development of weed species which are more hardy because of the selective advantage they may obtain upon expression of the new genes. Seed companies attempting to commercialize plants having insect resistance, virus resistance, fungal resistance, herbicide resistance, etc., each have had to address to governmental regulatory agencies and environmental interest groups the issues of outcrossing of these traits to related plant species. This concern has resulted in numerous meetings and workshops which discuss these issues (Serratos et al., 1997). The issues range from enhanced weediness to reduction in the biodiversity of the related wild relatives.

Gene flow from crops which are the products of traditional agricultural breeding have contributed to the weediness of their weedy relatives. Examples of these include: sugar beet, pearl millet, rice and rye. The ability to control gene flow to these related wild species has been limited by the lack of an effective method to control pollen production and the subsequent inability to restrict the distribution of the genes, if they indeed spread from cultivated plants to related wild species. Thus, there is an unmet need in the field of plant biotechnology for a method to selectively prevent the production of pollen for the

purpose of restricting the dissemination of recombinant genetic material, and to provide a means to select against wild plant relatives which have acquired the genetic material.

The horticultural industry which provides bedding plants for residential and commercial settings would have great interest in flowers which maintain their petals and/or color for longer periods of time. Flowers rapidly deteriorate after becoming pollinated. Inhibiting pollination, therefore, would add days or weeks to the useful life of many bedding plants used for the production of showy flowers. As an example of its importance, the horticulture industry currently supports a breeding program for male sterility in lilies for the purpose of preserving the flower shell and eliminating the production of lily pollen, which stains clothing and fabric. Similar efforts would also be advantageous in plants species such as Geranium species since these plants demonstrate petal shatter as early as 2 hours after pollination. Petal shatter can limit sales and crop commercialization. A broadly applicable genetic engineering approach to reduce pollen shed and lengthen flower petal life in these and numerous other species would provide an important new tool for the horticultural industry to produce products with improved characteristics.

N-phosphonomethylglycine, also known as glyphosate, is a well known herbicide that has activity on a broad spectrum of plant species. Glyphosate is the active ingredient of Roundup® (Monsanto Co.), an environmentally safe herbicide having a desirably short half life. When applied onto a plant surface, glyphosate moves systemically through the plant. Glyphosate is toxic to plants by inhibiting the shikimic acid pathway which provides a precursor for the synthesis of aromatic amino acids. Specifically, glyphosate affects the conversion of phosphoenolpyruvate and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimate synthase (EPSPS).

Through genetic engineering, it is possible to produce glyphosate tolerant plants by inserting into the plant genome a DNA molecule that causes the production of higher levels of wild-type EPSPS. Glyphosate tolerance can also be achieved by the expression of EPSPS variants which have lower affinity for glyphosate and therefore retain their catalytic activity in the presence of glyphosate (U.S. Patent No. 5,633,435). Enzymes which degrade glyphosate in the plant tissues (U.S. Patent No. 5,463,175) are also capable

of conferring cellular tolerance to glyphosate. Such genes, therefore, allow for the production of transgenic crops which are tolerant to glyphosate, thereby allowing glyphosate to be used for effective weed control with minimal concern of crop damage. For example, glyphosate tolerance has been genetically engineered into corn (U.S. Patent No. 5,554,798) and wheat (Zhou et al., 1995)

The use of glyphosate as a chemical gametocide has been described (U.S. Patent No. 4,735,649). Therein, it is disclosed that glyphosate can, under optimal conditions, kill about 95% of male gametes, while leaving about 40-60% of the female gametes capable of fertilization. In addition, a stunting effect was typically observed at the application levels disclosed, manifested by a reduction in the size of the plant and by a minor amount of chlorosis. Thus, a major drawback of using glyphosate as a gametocide, as is generally true with most gametocides, is the phytotoxic side effects resulting from lack of sufficient selectivity for male gametes.

SUMMARY OF THE INVENTION

In its broadest sense, the invention described herein provides a method to cause selective and regulated herbicide-induced ablation of specific cell types in plants. This method involves the insertion into the genome of a plant cell at least two distinct recombinant DNA molecules. A first DNA molecule comprises operably linked in the 5' to 3' orientation:

a first promoter that functions in plant cells to cause the production of a first RNA sequence;

a first DNA sequence encoding a first RNA sequence which encodes a protein that causes tolerance to a systemically translocated herbicide, preferably glyphosate;

a first 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the first RNA sequence.

Typically, the promoter used in the first DNA molecule is expressed in a constitutive fashion. Examples of suitable promoters that function effectively in this capacity include cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, figwort mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow

mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter and octopine synthase promoter.

The second DNA molecule of the invention comprises operably linked in the 5' to 3' orientation:

a second promoter that functions in plant cells to cause the production of a second RNA sequence;

a second DNA sequence encoding a second RNA sequence that is complementary to the first RNA sequence;

a second 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the second RNA sequence.

The promoter used in the second DNA molecule is not constitutively expressed. Rather, it has a more restricted expression pattern, preferably limited to one or more male reproductive tissues. Preferred promoters for use in the second DNA molecule of the invention include the TA29 tobacco tapetum-specific promoter, PA1 chalcone flavonone isomerase promoter, PA2 chalcone flavonone isomerase promoter, SLG promoter, LAT promoter, exopolygalacuronase promoter, Zmg13 promoter, LAT52 promoter, LAT59 promoter, and psgB6-1 promoter.

Expression of the first DNA molecule of the invention serves to generate tolerance to glyphosate in those tissues in which it is expressed. Expression of the second DNA molecule, on the other hand, causes the production of an RNA sequence which can inhibit the glyphosate tolerance generated by expression of the first DNA molecule. By using a promoter for the second DNA molecule which restricts the production of the antisense RNA to only a subset of the tissues which express the first DNA molecule, only the subset of tissues in which the second DNA molecule is expressed will be susceptible to glyphosate toxicity. In this way, a specific cell type or combination of cell types, depending upon the promoters utilized, can be selectively ablated by application of glyphosate to the plant.

Therefore, in accordance with one aspect of the present invention, there is provided a method for producing male sterile plants comprising the steps of inserting into the genome of plant cells the first and second DNA molecules of the invention; obtaining transformed plant cells containing the first and second DNA molecules; regenerating transformed plants from the transformed plant cells; and exposing the transformed plants to glyphosate.

According to another aspect of the invention, there is provided a method for the production of hybrid seed comprising effecting cross-fertilization of the disclosed male sterile plants with pollen from a male fertile donor, and harvesting seed from the progeny of the cross-fertilization.

In another aspect of the invention, there is provided a method of producing hybrid seed wherein the seed is capable of generating plants which have restored male fertility and which remain fertile upon application of glyphosate. Male sterile plants containing the first and second DNA molecules of the invention are generated as described herein. However, in this aspect of the invention, a male-fertile pollen parent is used which contains in its genome a third DNA molecule comprising operably linked in the 5' to 3' orientation:

a third promoter that functions in plant cells to cause the production of a third RNA sequence;

a third DNA sequence encoding a third RNA sequence which encodes a protein that causes tolerance to glyphosate;

a third 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the third RNA sequence;

wherein the third DNA molecule is not the same as the first DNA molecule.

The glyphosate tolerance gene of the third DNA molecule can be structurally unrelated to the glyphosate tolerance gene of the first DNA molecule. In this way, an antisense or cosuppression RNA molecule produced from the second DNA molecule can hybridize with the RNA produced by the first DNA molecule, thereby inhibiting its expression, but cannot hybridize to the glyphosate tolerance gene of the third DNA molecule due to lack of sufficient complementarily. Alternatively, the same or a similar glyphosate tolerance

gene can be used in the third DNA molecule as is used in the first DNA molecule. However, in this situation, there is a region of dissimilarity between the first and third DNA molecules which can be differentially targeted for inhibition by the second DNA molecule.

Further provided are the transformed plant cells, and plants regenerated therefrom, which contain the first, second and/or third DNA molecules of the invention.

Preferred plants used in the practice of the invention include, but are not limited to, corn, wheat, rice, canola, oat, barley, alfalfa, carrot, cotton, oilseed rape, sugar beet, sunflower, soybean, tomato, cucumber and squash.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 (SEQ ID NO:1) illustrates the DNA sequence of the P-Ztap promoter.

FIG. 2 (SEQ ID NO:2) illustrates the DNA sequence of the Petunia EPSPS intron

FIG. 3 (SEQ ID NO:3) illustrates the DNA sequence of the Petunia EPSPS chloroplast transit peptide

FIG. 4 (SEQ ID NO:4) illustrates the DNA sequence of the Petunia EPSPS intron/petunia EPSPS chloroplast transit peptide.

FIG. 5 pMON19470 and pMON19437 plasmid maps

FIG. 6. pMON19653 and pMON19340 plasmid maps

FIG. 7. pMON25232 plasmid map

FIG. 8. pMON25233 plasmid map

FIG. 9. pMON25234 and pMON25235 plasmid maps

FIG. 10. pMON25241 plasmid map

FIG. 11. Construction of pMON25258

FIG. 12. Construction of pMON25259

FIG. 13. Construction of pMON25260

FIG. 14. Relative CP4 expression in corn protoplasts co-electroporated with control or antisense vectors.

FIG. 15. Relative CP4 expression in wheat protoplasts co-electroporated with control or antisense vectors.

DETAILED DESCRIPTION OF THE INVENTION

RECOMBINANT DNA MOLECULES

Transcription of DNA into mRNA is regulated by the region of a gene referred to as the "promoter". The promoter region comprises a double stranded DNA sequence that signals RNA polymerase to associate with the sense and antisense DNA strands and to use the sense strand as a template to make a corresponding strand of mRNA complimentary to the sense DNA strand. This process of mRNA production using a DNA template is referred to as gene "expression" or "transcription".

The particular promoters selected for use in embodiments of the present invention should be capable of causing the production of sufficient expression to, in the case of the first DNA molecule, generate glyphosate tolerance, and in the case of the second DNA molecule, inhibit the glyphosate tolerance to a sufficient degree to render the tissues sensitive to glyphosate toxicity.

The first DNA molecule will typically contains a constitutive promoter, a structural DNA sequence encoding a glyphosate tolerance enzyme, and a 3' non-translated region. A number of constitutive promoters which are active in plant cells have been described. Suitable promoters for constitutive expression of glyphosate tolerance for the first DNA

molecule include, but are not limited to, the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985), the Figwort mosaic virus (FMV) 35S (Sanger et al., 1990), the sugarcane bacilliform virus promoter (Bouhida et al., 1993), the commelina yellow mottle virus promoter (Medberry and Olszewski, 1993), the light-inducible promoter from the small subunit of the ribulose-1,5-bis-phosphate carboxylase (ssRUBISCO) (Coruzzi et al., 1984), the rice cytosolic triosephosphate isomerase (TPI) promoter (Xu et al., 1994), the adenine phosphoribosyltransferase (APRT) promoter of *Arabidopsis* (Moffatt et al., 1994), the rice actin 1 gene promoter (Zhong et al., 1996), and the mannopine synthase and octopine synthase promoters (Ni et al., 1995). All of these promoters have been used to create various types of plant-expressible recombinant DNA constructs. Comparative analysis of constitutive promoters by the expression of reporter genes such as the uidA (β-glucuronidase) gene from *E. coli* has been performed with many of these and other promoters (Li et al., 1997; Wen et al., 1993).

Promoters used in second DNA molecule are selected to confer specific expression where cell lethality is desired. In a preferred embodiment, the promoter will be capable of directing expression exclusively or primarily in a tissue critical for pollen development such as the pollen itself, the tapetal cell layer of the anther, or the anther tissues.

Plant promoters capable of regulating the expression of genes in particular cell and tissue types are well known. Those that are most preferred in the embodiments of this invention are promoters which express specifically during the development of the male reproductive tissue or in pollen at levels sufficient to produce inhibitory RNA molecules complementary to the sense RNA transcribed by the constitutive promoter of the first DNA molecule. Examples of these types of promoters include the TA29 tobacco tapetum-specific promoter (Mariani et al., 1990), the PA1 and PA2 chalcone flavonone isomerase promoters from petunia (van Tunen et al., 1990), the SLG gene promoter from *Brassica oleracea* (Heizmann et al., 1991), and LAT gene promoters from tomato (Twell et al., 1991).

Anther and pollen-specific promoters from rice have been isolated. Examples include the Osg6B promoter, which was shown to drive expression of the β-glucuronidase gene in transgenic rice in immature anthers. No activity was detected in other tissues of spikelets,

leaves or roots (Yokoi et al., 1997). The PS1 pollen-specific promoter from rice has been shown to specifically express the β-glucuronidase gene in rice pollen (Zou et al., 1994). Additional rice genes have been identified that specifically express in the anther tapetum of rice (Tsuchiya et al., 1994; Tsuchiya et al., 1997). The isolation of additional genes expressed predominantly during anther development in rice can be performed, for example, by construction of a cDNA library to identify anther specific clones (Qu et al., 1997).

Those skilled in the art are aware of the approaches used in the isolation of promoters from genes or members of gene families that are highly expressed in pollen, or in plant cell types involved in the production of pollen (Stinson et al., 1987; Brown and Crouch, 1990; McCormick et al., 1989). Further examples of these promoters include the promoter for the exopolygalacturonase gene of maize (Dubald, et al., 1993) and the promoter for the Zmc13 mRNA (Hanson et al., 1989). Promoters which have been shown to preferentially express in tomato pollen are the LAT52 and LAT59 promoters (Twell et al., 1991). The entire sequence of the maize pZtap promoter is disclosed in SEQ ID NO:1. A portion of this sequence (psgB6-1) was disclosed in U.S. Patent No. 5,470,359.

A recombinant DNA molecule of the invention typically comprises a 5' non-translated region, a promoter, a DNA sequence of a plant intron, a structural sequence encoding a chloroplast transit peptide (CTP), a DNA coding sequence for a glyphosate tolerance gene, and a 3' non-translated region.

The 5' non-translated leader sequence can be derived from the promoter selected to express the heterologous DNA sequence, and can be specifically modified if desired so as to increase translation of mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence which accompanies the promoter sequence. The leader sequence could also be derived from an unrelated promoter or coding sequence.

The 3' non-translated region of a recombinant DNA molecule contains a polyadenylation signal which functions in plants to cause the addition of adenylate nucleotides to the 3'

end of the RNA. The 3' non-translated region can be obtained from various genes which are expressed in plant cells. The nopaline synthase 3' untranslated region (Fraley et al., 1983), the 3' untranslated region from pea ssRUBISCO (Coruzzi et al., 1994), the 3' untranslated region from soybean 7S seed storage protein gene (Schuler et al., 1982) and the pea small subunit of the pea ssRUBISCO gene are commonly used in this capacity. The 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes are also suitable.

Examples of plant introns suitable for expression in monocots includes, for example, maize hsp70 intron, rice actin 1 intron, maize ADH 1 intron, *Arabidopsis* SSU intron, *Arabidopsis* EPSPS intron, petunia EPSPS intron and others known to those skilled in the art.

Examples of CTPs suitable to direct the targeting of the glyphosate tolerance gene product to the chloroplast of the plant cell include the petunia EPSPS CTP, the *Arabidopsis* EPSPS CTP2 intron and others known to those skilled in the art.

GLYPHOSATE TOLERANCE GENES

Various approaches have been developed whereby it is possible to express a heterologous DNA sequence, frequently hereinafter referred to as a glyphosate tolerance gene or glyphosate tolerance coding sequence, in transgenic plants in order to confer upon the plants tolerance to the herbicide glyphosate. Any such glyphosate tolerance genes known to the skilled individual are suitable for use in the practice of the present invention.

Glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids, plant hormones and vitamins. Specifically, glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). For the purposes of the present invention, the term "glyphosate" should be considered to include any herbicidally active form of N-phosphonomethylglycine (including any salt thereof) and other forms which result in the production of the glyphosate anion in plants.

A variety of native and variant EPSPS enzymes have been expressed in transgenic plants in order to confer glyphosate tolerance (Barry et al., 1992), any of which can be used in the

invention. Examples of some of these EPSPS include those described and/or isolated in accordance with U.S. Patent Nos. 4,940,835, 4,971,908, 5,145,783, 5,188,642, 5,310,667, and 5,312,910, which are incorporated herein by reference. They can also be derived from a structurally distinct class of non-homologous EPSPS genes, such as the class II EPSPS genes isolated from *Agrobacterium* sp. strain CP4 as described in U.S. Patent Nos. 5,633,435 and 5,627,061 which is also incorporated herein by reference. Alternatively, a glyphosate degrading enzyme could be used to confer glyphosate tolerance, for example using a glyphosate oxidoreductase gene as described in U.S. Patent No. 5,312,910, which is incorporated herein by reference.

A double stranded DNA molecule of the present invention can be inserted into the genome of a plant by any suitable method. Suitable plant transformation methods include *Agrobacterium*-mediated transformation, the use of liposomes, electroporation, chemicals that increase free DNA uptake, free DNA delivery via microprojectile bombardment, transformation using viruses or pollen, etc.

After transformation of cells (or protoplasts), choice of methodology for the regeneration step is not critical, with suitable protocols being available for hosts from *Leguminosae* (alfalfa, soybean, clover, etc.), *Umbelliferae* (carrot, celery, parsnip), *Cruciferae* (cabbage, radish, rapeseed, etc.), *Cucurbitaceae* (melons and cucumber), *Graminae* (wheat, rice, corn, etc.), and *Solanaceae* (potato, tobacco, tomato, peppers).

Such methods for plant transformation and regeneration are well known and readily available to the skilled individual in this art (for example, see Hinchee et al., (1994), and Ritchie & Hodges (1993) for reviews).

MALE STERILITY AND HYBRID SEED PRODUCTION

One embodiment of the present invention relates to an improved method for producing male sterile plants wherein glyphosate is used as a gametocide. The effective use of glyphosate in this capacity, and the use of gametocides in general, has been limited by the lack of sufficiently high selectivity for male gametes so as to prevent unnecessary damage to female gametes and vegetative tissue.

The present invention exploits the use of glyphosate tolerance genes, in combination with the tissue-specific RNA-mediated inhibition of gene expression (e.g., by antisense, cosuppression, ribozymes, etc.), to provide improved methods for glyphosate-induced male sterility. The techniques described herein are applicable to essentially all crop species, including monocotyledons such as rice, wheat, oats, barley, corn and the like, as well as dicotyledons such as alfalfa, canola, carrot, cotton, oil seed rape, sugar beet, sunflower, soybean, tomato, cucumber and melons, squash, and others. This method can be applied to ornamental plants of many varieties to engineer the plants for hybrid seed production.

Two crops of particular interest are corn (*Zea mays*) and wheat (*Triticum aesativum*). Mechanical emasculation is currently the most common method used for the production of male sterility in corn, and application of chemical gametocides is the most common method used for wheat. The method describe herein represents an effective and efficient alternative means for the production of hybrid seed in these and other crops.

In order to render male reproductive tissue selectively susceptible to glyphosate-induced toxicity, despite the constitutive expression of the glyphosate tolerance enzyme encoded by the first DNA molecule, a second DNA molecule is used. The second DNA molecule contains a tissue-specific promoter which directs expression exclusively or primarily in male reproductive tissue of a DNA sequence which causes the production of an RNA sequence. This RNA sequence is complementary to and capable of hybridizing with the RNA produced by the first DNA molecule, thereby inhibiting expression of the protein encoded by the first DNA molecule via antisense or cosuppression mechanisms (see for example, Schuch, 1991; Bird, 1991; Jorgensen, 1990). Alternatively, the RNA can encode a catalytic RNA molecule (i.e., a ribozyme) engineered to cleave the mRNA produced from the first DNA molecule (see for example, Gibson, 1997; Steinecke, 1994; Marrs, 1995). In this way, glyphosate tolerance is selectively attenuated in a male-specific manner by the tissue-specific inhibition of expression of the first DNA molecule.

The second DNA molecule can target the coding sequence of the glyphosate resistant gene of the first DNA molecule. Alternatively, other regions of the first DNA molecule can be targeted, such as intron sequences and/or CTP coding sequences, etc.

The skilled individual will recognize that numerous approaches can be used to arrive at a transgenic plant containing the first and second DNA molecules of the invention. The DNA molecules can be introduced into a plant in any appropriate manner and/or order, e.g., simultaneously, separately, sequentially etc. For example, where the first and second DNA molecules are introduced separately to produce independent lines, the two plant lines can be crossed using traditional breeding methods and progeny from the cross assayed for the presence of the transgenes. The progeny containing both transgenes are allowed to self and progeny from this self can be assayed for the presence of both transgenes. Those populations which are homozygous for both genes are tested for response to glyphosate application on male sterility and vegetative glyphosate tolerance. Lines exhibiting effective vegetative tolerance to glyphosate and demonstrating the desired level of male sterility are further propagated.

Alternatively, the expression cassettes comprising the first DNA molecule and the second DNA molecule may be contained on the same plasmid, and transformed into the plant cells as a single piece of DNA. Regenerated plants produced from cells so transformed can be treated with glyphosate and those exhibiting the desired level of glyphosate tolerance and the desired level of male sterility are pollinated with wild type pollen, and the seed collected. Seed from this cross can be germinated and plants assayed for the presence of both genes. Positive plants are allowed to self and the seed collected. A subset of the collected seed is planted, assayed for the presence of both genes, and treated with glyphosate. The plants are scored for the desired level of glyphosate tolerance and male sterility. Sibling seed are planted and propagated for seed increase.

Plants regenerated from transformed plant cells comprising the first and second DNA molecules of the invention are vegetatively- and female-tolerant of glyphosate, but are male-sensitive to the compound. In the absence of glyphosate spray, the plants are normal and fully fertile. This allows for very straightforward line maintenance via selfing. When glyphosate is sprayed on the plants of the present invention, complete male sterility can result.

The disclosed method for generating male-sterile plants is readily adapted to the production of hybrid seed, including hybrid seed with restored fertility. Thus, relating to

an additional embodiment of the present invention, there is provided a method of producing hybrid seed which comprises first regenerating a plant from a transformed plant cell which contain the first and second DNA molecules described above, increasing the number of plants by allowing growth and self-fertilization in the absence of glyphosate, exposing the plants to glyphosate to produce male sterile plants, effecting cross fertilization with pollen from a suitable donor, and harvesting seed from the progeny of the cross-fertilization. During production of hybrid seed, the seed parent plants are sprayed with glyphosate and rendered male sterile and pollinated by the pollen parent which are male fertile. Thus, hybrid seed produced in this way will generate plants with restored male fertility as long as glyphosate is not applied to them. Application of glyphosate to hybrid plants containing the first and second DNA molecules of the present invention would render them male sterile for the above-discussed reasons.

In another embodiment of the present invention, there is provided a method of producing hybrid seed, wherein the seed is capable of generating plants which have restored male fertility and which remain fertile upon application of glyphosate. Male sterile plants are generated as discussed above. However, in this embodiment, the male-fertile pollen parent contains in its genome a third DNA molecule which comprises a constitutive promoter and a structural DNA sequence that causes the production of a protein capable of conferring tolerance to glyphosate, wherein said tolerance is not substantially affected by the RNA encoded by the second DNA molecule. Thus, the third DNA molecule can express, for example, a glyphosate tolerance gene under the control of a constitutive promoter, wherein the ability of the gene to confer glyphosate tolerance is not affected by the antisense RNA produced from the second DNA molecule.

The glyphosate tolerance gene of the third DNA molecule can be structurally unrelated (i.e., lacking significant homology with the glyphosate tolerance gene of the first DNA molecule). In this way, an antisense or cosuppression RNA molecule produced from the second DNA molecule can hybridize with the RNA produced by the first DNA molecule, thereby inhibiting its expression, but cannot hybridize to the glyphosate tolerance gene of the third DNA molecule due to lack of sufficient complementarity. Nonhomologous glyphosate tolerance gene combinations suitable for this embodiment can include, for example, class I and class II EPSPS genes (see for example, U.S. Patent No. 5,633,435), or

any other combination wherein both genes provide glyphosate tolerance when expressed in plants but are sufficiently nonhomologous so that the second DNA molecule inhibits the expression of one but not the other. A gene encoding a glyphosate degrading enzyme could also be used in either the first or third DNA molecule, while using, for example, an EPSPS in the other.

Alternatively, the same or a similar glyphosate tolerance gene can be used in the third DNA molecule as is used in the first DNA molecule. However, in this situation, there will be a region of dissimilarity between the first and third DNA molecules which can be differentially targeted by the second DNA molecule. Thus, although the first and third DNA molecules may utilize an identical glyphosate tolerance gene, they would have differences, for example in their untranslated regions, that could be selectively targeted. For example the third DNA molecule could be designed to contain a region, such as an intron sequence or a CTP sequence, distinct from the region of the first DNA molecule that is targeted for inhibition by the second DNA molecule.

PREVENTS OUTCROSSING TO WILD RELATIVES

It will be appreciated by those skilled in the art that the methods and compositions of the invention can be used to prevent the production of viable pollen in plants where it is desired to limit the distribution of the glyphosate tolerance genes to related wild species. For example, this would be advantageous with turf grasses where constitutive vegetative glyphosate resistance is a desirable characteristic, but outcrossing to wild grass species is not. The application of glyphosate to fields containing plants of this invention will provide environmentally safe weed control while at the same time limiting the possibility of outcrossing of the glyphosate tolerance genes to wild species.

The invention can also be applied to forestry trees (Strauss et al. 1995) such as popular, Douglas fir, Eucalyptus, Loblolly pine, Radiata pine, Southern pine, and Sweetgum. Trees so produced will be glyphosate tolerant and will become pollen sterile when glyphosate is applied at the appropriate developmental stage, thereby limiting the spread of viable pollen.

Another application of the invention relates to minimizing outcrossing of rice with the weed species "red rice". Any potential concern of the escape of glyphosate tolerance to weedy species will be significantly reduced since the promoter of the second DNA molecule, preferably a tapetal specific promoter, is likely to function in compatible weed species. As such, any resulting progeny of an outcross event would have male gametes or associated cells sensitive to the toxic effects of glyphosate.

Outcrossing of *Brassica napus* (canola) to *Brassica rapa* and *Brassica juncea* has been demonstrated under field conditions. Application of glyphosate to a hybrid weed species, which by outcrossing from a transgenic crop plant contains the first and second DNA molecules of the invention, would render the plants male sterile and severely limit their ability to survive and/or distribute the glyphosate tolerance trait after treatment with glyphosate.

Furthermore, crops in which the vegetative parts of the plant are the primary agricultural product such as sugarbeet, sugarcane, potatoes, sweet potatoes; leafy vegetables such as lettuce, cabbage, spinach, and tea; vegetable root crops such as carrots, radish, turnips, garlic and onions would be vegetatively resistant to the toxic effects of glyphosate when produced according to the present invention. Hence, glyphosate could be used to control weeds in these crops and the plants, once sprayed, would become male sterile.

PREVENTS SEED PRODUCTION FROM VOLUNTEER PLANTS

Volunteer crop plants are plants which occur in or around the field where the previous season's crop was produced. In some situations, the volunteer crop plants escape into the environment and become weeds. Where such crops contain glyphosate tolerance genes, the possibility of the potential dissemination of glyphosate tolerance genes into the environment is of concern. However, such concerns would be minimized if a method were available which could effectively limit this possibility.

The invention described herein can prevent or severely limit seed production in volunteer crop plants sprayed with glyphosate and thereby prevent the propagation of glyphosate tolerant weedy plants. Canola (*Brassica napus*) has been of particular concern where the winter oil seed rape varieties in Europe have become weeds in and around the areas where

it is commonly cultivated. *Brassica napus* seed can remain in the soil profile and produce volunteer plants in subsequent crop rotations. The use of the methods of this invention for the hybrid production of canola would lessen environmental concerns since the plants would be rendered male sterile when sprayed with glyphosate.

The following examples are included to demonstrate examples of certain preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from scope of the invention.

EXAMPLES

Example 1

Inhibition of CP4 expression by different antisense gene fragments in corn protoplasts and wheat protoplasts

A. Preparation of plasmids pMON19340, pMON25232, pMON25233, pMON25234, pMON25235 and pMON25241

Plasmid pMON19470, described in U.S. Patent No. 5,424,412 contains the 0.65 kb cauliflower mosaic virus (CaMV) 35SRNA promoter (e35S) containing a duplication of the -90 to -300 region (Kay et al., 1987), the first intron from the maize HSP70 gene (U.S. Patent 5,424,412), a multicloning site, and a 0.25kb fragment containing the 3' polyadenylation sequences from the nopaline synthase (NOS) gene (Fraley et al., 1983) in a pUC119 backbone (Yanisch-Perron et al., 1985). pMON19340, pMON25232, pMON25233, pMON25234, pMON25235 and pMON25241 were all derived from pMON19470 expression cassette components.

Figure 6. pMON19653 e35S/hsp70 intron/CTP2/CP4 EPSPS/NOS3'

Figure 6. pMON19340 e35S/PetEPSP intron/PetEPSPS CTP/CP4/NOS3'

Figure 7. pMON25232 e35S/hsp70 intron/antiPetEPSPS intron-PEPSP CTP/NOS3'

Figure 8. pMON25233 e35S/hsp70 intron/antiPetEPSPS intron/NOS3'

Figure 9. pMON25234 e35S/hsp70 intron/antiPetEPSPS CTP/NOS3'

Figure 9. pMON25235 e35S/hsp70 intron/anti-CP4 EPSPS/NOS3'

Figure 10. pMON25241 e35S/hsp70 intron/anti-GUS/NOS3'

B. Analysis of gene expression in corn protoplasts

Expression analysis of genes in plant protoplasts has been well documented (Schledzewski et al., 1994; Steinbiss et al., 1991; Stefanov et al., 1991). Protoplast expression analysis is often a useful means of predicting if certain genes will function in plant cells. A corn protoplast transient expression system was used to evaluate the effects of different antisense gene fragments on CP4 EPSPS expression. Corn leaf protoplast isolation and electroporation was performed as described by Sheen, 1991. Plasmid DNAs were prepared by using standard alkaline lysis followed by cesium chloride gradient purification (Maniatis et al., 1982). Five μg of pMON19340 DNA with 40 μg of one of the four antisense plasmid DNAs (pMON25232, pMON25233, pMON25234, and pMON25235) and a GUS (β-*gluc*) antisense control plasmid (pMON25241) were concomitantly introduced into corn protoplasts by electroporation. pMON25241 DNA plasmid was used as a filler to obtain the same total amount of plasmid used in the electroporation for CP4 EPSPS DNA-only control and 5 μg LUX plasmid DNA (pMON19437) control. Cells after electroporation were incubated for 24 hours and then collected by centrifugation. Total protoplast protein was harvested by three cycles of freeze/thaw followed by centrifugation to remove the cell debris. Total protein concentration was determined by Bio-Rad protein analysis (Bio-Rad Laboratories, Cat# 500-0006).

CP4 EPSPS expression was quantitated by ELISA. The crude protoplast extract containing 1 mg total protein was added to goat anti-CP4 EPSPS IgG coated wells of a 96-well plate for reaction. A second antibody, rabbit anti-CP4 EPSPS IgG, was added to the

plate after washing and the plate allowed to incubate overnight. After washing, alkaline phosphatase conjugated donkey anti-rabbit IgG was added to each well and the presence of CP4 EPSPS visualized with alkaline phosphatase substrate. Quantitation of sample CP4 EPSPS concentration was accomplished by extrapolation of the logistics curve fit of the CP4 EPSPS standard curve present on each plate. Luciferase analysis was performed as described in US Patent No. 5,424,412.

The effect of each antisense is represented by the ratio of CP4 EPSPS expression over the level of expression of the luciferase internal control (CP4 EPSPS/LUX). As shown in Figure 14 and Table 1, each of the antisense versions decreases expression of CP4 EPSPS. Interestingly, the anti-intron construct, pMON25232 appears to reduce CP4 EPSPS levels as well as the construct containing the entire CP4 EPSPS coding sequence in antisense orientation.

Table 1. Effect of CP4 EPSPS antisense fragments on CP4 EPSPS expression in maize protoplasts.

Vector	Gene Expressed	CP4/LUX
control	no DNA	0
pMON19340	CTP4-CP4 EPSPS	100 X
pMON25235	CTP4-CP4 + antiPetEPSPS intron-anti CTP4	33 X
pMON25232	CTP4-CP4 + antiPetEPSPS intron	24 X
pMON25233	CTP4-CP4 + anti-CTP4	63 X
pMON25234	CTP4-CP4 + anti-CP4	22 X

C. Analysis of gene expression in wheat protoplasts

Electroporation of protoplasts from Bobwhite wheat was conducted with the control and antisense constructs. Protoplasts were made from suspension cultures by a modified protocol of (Zhou et al., 1993) and (He et al., 1994). Briefly, 8 g of wheat cell suspension was resuspended with 40 mL enzyme solution and incubated at 26°C for 2 hours on a rotator at 40 rpm. The centrifugation of the solution was conducted at 200g for 8 minutes. The protoplasts were washed twice with centrifugation between each wash. They were resuspended in 10 mL wash solution and stored on ice. The number of protoplasts were determined and the volume adjusted to make the concentration 4 X 10^6 protoplasts/mL. Protoplasts (0.75 mL) were added to each electroporation cuvette, then up to 50 µg

plasmid DNA in 50 µL solution as added to the protoplasts. The electroporation conditions using a Bio-Rad Gene Pulser were 960 µFarads and 160 volts. The samples remained on ice for 10 minutes, and were then pipetted into MS1 WSM media and incubated in the dark for 18-22 hours at 24°C. Cells were pelleted by centrifugation at 200-250 g for 8 minutes. The pellets were frozen on dry ice.

The ELISA (enzyme linked immunosorbent assay) procedure used to quantitate CP4 Enol-Pyruvyl-Shikimate-3-Phosphate Synthase (EPSPS) in corn leaf, seed, and whole plant tissues. The assay described in this procedure is a direct ELISA that quantitates the levels of CP4 EPSPS protein present in corn plant tissue extracts. The corn tissue is extracted in 20:1 volume/weight of buffer in a Brinkmann polytron mechanical homogenizer at 17,500 rpm for 30 seconds. A single centrifugation step at 6,660 g for 8 minutes separates the insoluble debris from the soluble extract. The levels in plant samples are compared to a purified reference standard of CP4 EPSPS isolated from *Escherichia coli*. In brief, 96-well polystyrene plates are coated with purified goat anti-CP4 (2 µg/well) then blocked with non-fat dry milk (1% in 1XPBST buffer, phosphate buffered saline pH 7.4, 0.05% Tween-20) for 30 minutes at 30°C, then washed 3 times with 1XPBST. Two hundred-fifty µL of soluble plant tissue extract/well (the extract may be diluted with 1XPBST as needed) is added to the antibody-coated wells alongside a concentration range of pure CP4 EPSPS protein standards. The plates are incubated allowing antigen capture by the surface bound antibodies. The unbound sample is washed away with buffer and rabbit anti-CP4 EPSPS conjugated to horseradish peroxidase (1:170 in 1XPBST) is added, binding to the CP4 EPSPS antigen. Following incubation and washing, peroxidase substrate is added to each well. Wells containing CP4 EPSPS and hence, the antibody sandwich (goat anti-CP4 EPSPS + plant CP4 EPSPS + rabbit anti-CP4 EPSPS horseradish peroxidase), will turn blue. The peroxidase TMB substrate and hydrogen peroxide buffers (cat# 50-76-02, Kirkegaard & Perry Labs) reaction results in a soluble blue product and when the reaction is stopped with 3M phosphoric acid, the product turns yellow. Quantitation of sample CP4 EPSPS concentration is accomplished by extrapolation (based on sample absorbance value obtained from and ELISA plate reader, read at 450 nm with a reference wavelength of 655 nm) from the log-log quadratic regression curve fit of the CP4 EPSPS standard curve ranging from (0.1ng - 2.0 ng CP4/well) or (0.4ng- 8.0 ng CP4/mL).

The results shown in Table 2 and Figure 15 demonstrate that in wheat protoplasts, it is possible to inhibit glyphosate tolerance gene expression from pMON19340 using antisense to the genetic elements expressed from the plant expression vectors pMON25235, pMON25232, pMON25233, and pMON25234. The plant expression vector pMON19340 constitutively transcribes an RNA molecule containing a 5' leader sequence, an intron, an exon sequence comprising a RNA sequence encoding a CTP and a glyphosate tolerance gene, and a 3' untranslated region. The antisense to the intron/exon sequence provided unexpected synergy in the inhibition of expression of CP4 EPSPS protein in the wheat protoplast assay.

Table 2. Effect f antisense constructs n CP4 EPSPS expression in wheat protoplasts

		antisense fragment	OD450	OD450	mean	blank	Luc	CP4/ Luc	ave	STDEV	Rela -tive CP4
1	19340	no antisense control	0.53	0.52	0.525	0.454	16.65	0.0273	0.039	0.01312	
2	19340	no antisense control	0.311	0.35	0.3305	0.2595	4.88	0.0532			
3	19340	no antisense control	0.514	0.54	0.527	0.456	12.45	0.0366			
4	19340	anti-GUS control	0.546	0.557	0.5515	0.4805	67.81	0.0071	0.007	0.00008	100 %
5	19340	anti-GUS control	0.571	0.584	0.5775	0.5065	73.17	0.0069			
6	19340	anti-GUS control	0.558	0.569	0.5635	0.4925	69.99	0.0070			
7	19340 + 25232	anti-PEPSP intron/CTP	0.11	0.099	0.1045	0.0335	75.47	0.0004	0.0002	0.00024	4.0%
8	19340 + 25232	anti-PEPSP intron/CTP	0.057	0.077	0.067	-0.004	112.9	0.0000			
9	19340 + 25232	anti-PEPSP intron/CTP	0.09	0.088	0.089	0.018	112.6	0.0002			
10	19340 + 23233	anti-PEPSP intron	0.5	0.4	0.45	0.379	75.07	0.0050	0.005	0.00164	79.1 %
11	19340 + 23233	anti-PEPSP intron	0.559	0.519	0.539	0.468	63.38	0.0074			
12	19340 + 23233	anti-PEPSP intron	0.564	0.467	0.5155	0.4445	105.3	0.0042			
13	19340 + 25234	anti-PEPSP CTP	0.503	0.458	0.4805	0.4095	102	0.0040	0.004	0.00025	60.0 %
14	19340 + 25234	anti-PEPSP CTP	0.579	0.508	0.5435	0.4725	114.9	0.0041			
15	19340 + 25234	anti-PEPSP CTP	0.522	0.518	0.52	0.449	100.2	0.0045			
16	19340 + 25235	anti-CP4	0.428	0.417	0.4225	0.3515	109.2	0.0032	0.003	0.00095	44.0 %
17	19340 + 25235	anti-CP4	0.444	0.453	0.4485	0.3775	95.02	0.0040			
18	19340 + 25235	anti-CP4	0.308	0.313	0.3105	0.2395	115.1	0.0021			
19	Blank		0.087	0.088	0.0875	0.0165	0				
20	Blank		0.056	0.055	0.0555	-0.0155	0				

Example 2

Production of stably transformed corn plants with anther specific promoter constructs.

A. Preparation of pMON25258, pMON25259, and pMON25260

A maize genomic clone was provided by S. Goff, USDA, Albany, CA, which contained the coding region of an anther specific gene (SGB6) from maize and 2,719 nucleotide base pairs (pSGB6) of the 5' upstream promoter region of the gene. The DNA containing the SGB6 promoter region (pSGB6) was digested with the restriction endonuclease NheI, following manufacturers instructions (New England BioLabs), the NheI 3' overhang was filled in with complementary nucleotides by Klenow polymerase (New England BioLabs) by the method described by Maniatis et al. 1982. The DNA fragment containing the pSGB6 promoter region was further digested with HindIII to produce a 2,656 base pair fragment of the SGB6 upstream promoter region. This 2,656 nucleotide base pair SGB6 promoter fragment is called P-Ztap for Promoter *Zea* Tapetal. The P-Ztap promoter was then inserted into pMON19648 which had been digested with the endonuclease BglII, the overhanging ends of the DNA filled with complementary nucleotides by Klenow polymerase, then further digested with the endonuclease HindIII. The P-Ztap promoter fragment was ligated with the digested pMON19648 vector fragment using T4 DNA ligase (New England BioLabs) by the method described in Maniatis et al. 1982. The P-Ztap promoter replaces the E35S promoter region in this plasmid to yield pMON25258 (P-ZTap/HSP70 intron/GUS/NOS 3') (Figure 11.). The gene GUS and GUS:1, refers to β-glucuronidase (β-gluc), which is a scorable marker often used in transgenic plants to determine tissue specific expression (Jefferson et al. 1987). Similarly, the P-Ztap promoter was then inserted into pMON25235 which had been digested with BglII, the overhanging end filled with complementary nucleotides by Klenow polymerase then the plasmid digested with HindIII. The P-Ztap promoter replaces the E35S promoter region to yield pMON25259 (P-Ztap/HSP70 intron/anti-CP4 EPSPS /NOS 3') (Figure 12). The 5.4Kb expression cassette was isolated from pMON25259 contained on a KpnI/PvuII DNA fragment. This DNA fragment was then ligated using T4 DNA ligase into pMON25258 in which the plasmid had been previously digested with HindIII, the end

filled with complementary nucleotides by Klenow polymerase, then digested with KpnI. The resulting plasmid is pMON25260 which contains P-Ztap/HSP 70 intron/GUS/NOS 3' :: P-Ztap/HSP 70 intron/anti-CP4 EPSPS/NOS 3' (Figure 13).

B. Production and identification of transformed corn plants

pMON25260 DNA was co-transformed into Hi-II corn plant with pMON19653 DNA (E35S/Zmhsp70 intron/PetCTP2/CP4 EPSPS/NOS 3') by bombarding embryogenic corn tissue culture cells using a biolistic particle gun as described by Brown et al (U.S. Patent No. 5,424,412). Transformed cells were selected for glyphosate resistance and whole plants were regenerated and grown under greenhouse conditions.

Transgenic plants containing the CP4 EPSPS antisense gene were detected by a rapid Polymerase Chain Reaction (PCR) screening method. Twenty milligrams (mg) of leaf tissue from young corn seedlings was collected in a 1.5 milliliter (mL) microcentrifuge tube, frozen on dry ice, then pulverized into powder with a wooden applicator stick. Five hundred microliters (μL) of extraction buffer (100 mM Tris buffer, pH8.0; 50 mM EDTA; 500 mM NaCl; 10 mM 2-mercaptoethanol) was added to the tube and the tube was boiled for 10 minutes in a water bath. The extract was centrifuged (12,000 rpm, 10 minutes) in a tabletop microcentrifuge and the supernatant transferred to a new tube, then 50 μL of 10M ammonium acetate plus 1 mL 95% ethanol was added. After 5 minutes at room temperature, the DNA was pelleted by centrifugation of the tube at 12,000 rpm at room temperature for 10 minutes. Twenty-five μL of TE buffer (10 mM Tris buffer, pH 8.0; 1 mM EDTA) was used to resuspend the DNA pellet. Contaminating RNA was destroyed by adding 0.5 μL 10 mg/mL RNAse A to the DNA solution and incubating the tube for 5 minutes at 37°C. One μL of the extract was used to perform PCR reactions using the PCR Core Kit (Boehringer Mannheim, Cat. #1578553) and following the method described in this Kit. The DNA primers used in the PCR for detecting the CP4 EPSPS antisense expression cassette were SEQ ID NO:5 (5'- GAACAAGTTCATGAGCAAGGACCCTG - 3') located in the P-Ztap promoter and SEQ ID 6 (5'- CAAGCTCAATGGCGTGGATTGCG - 3') located in the CP4 EPSPS antisense gene. A Perkin Elmer thermocycler was used with the following conditions:

One cycle: 94°C, 3 minutes; 64°C, 1 minute; 72°C, 3 minutes

40 cycles: 94°C, 1 minute; 64°C, 30 seconds; 72°C, 3 minutes

Corn lines were determined to be transgenic for this antisense CP4 EPSPS expression cassette if they were positive for the presence of an ~1.3 kilobase (kb) DNA fragment detected on an agarose gel by following the methods of Maniatis et al.(1982). In separate reactions, specific primers were used to detect the GUS (β-*gluc*) expression cassette regions of pMON25260. The primers for detecting the GUS expression cassette were SEQ ID NO:5 (5'- GAACAAGTTCATGAGCAAGGACCCTG - 3)' located in the P-Ztap promoter sequence and SEQ ID NO:7 (5' - GTAGAGCATTACGCTGCGATGG - 3') located in the middle of the β-*gluc* coding region. An ~1.5 kb DNA fragment was observed by agarose gel electrophoresis in corn lines that received this region of pMON25260. Seventeen corn lines were determined to be PCR positive for the antisense CP4 EPSPS gene (Table 3).

Example 3

Evaluation of expression from pZtap promoter

Histochemical localization of GUS activity was used to evaluate the expression pattern from the P-Ztap promoter. This method was performed essentially as described by Van der Krol et al. (1991). Before staining, anthers, ovaries and other plant tissues were cut into two halves with a razor blade to allow X-gluc substrate to penetrate the tissue. To exclude artifacts which can result from differences in cell size, penetration of substrate into the tissue, and background activity, several independent histochemical assays were performed on anthers of both transgenic and nontransgenic plants.

Sixteen out of 43 transgenic Hi-II lines produced strong levels of GUS activity in anthers by histochemical staining. Five of the GUS-positive lines showed specific GUS staining only in anthers. Seven lines showed male-specific GUS staining in other tassel tissues such as glumes, lemma, palea, and pollen grains in addition to anther staining. Four lines showed expression in ovary and leaf, but the staining was significantly weaker than in tassel tissues.

Anti-CP4 EPSPS RNA produced from pMON25260 was detected in the Hi-II lines using a RT-PCR assay. Total RNA was extracted from tassel tissues as described above. First

strand cDNA was produced using reverse transcriptase reactions carried out with the Stratagene RT-PCR Kit (Cat. #200420) according to the manufacturer's instructions. One μg total RNA and 2.5 pmol of primer SEQ ID NO:8 (CP42 : 5'- CGA GGA CGT CAT CAA TAC GGG CAA GGC- 3') were used in each 20 μL reverse transcription reaction. PCR reactions were then performed on 1 μL of cDNA sample using the PCR Core Kit (Boehringer Mannheim, Cat. #1578553) using 300 nM each of SEQ ID NO:8 and SEQ ID NO:9 (5'- CAC GTC GAT GAC TTG GCC GGT GAG C -3') as primers in a 100 μL reaction volume and the using the following thermocycle conditions:

One cycle of: 94°C, 3 minutes; 64°C, 1 minute; 72°C, 3 minutes;
30 cycles of: 94°C, 1 minute; 64°C, 30 sec; 72°C, 3 minutes

Using this technique, 17 of 30 transgenic Hi-II lines evaluated showed detectable antisense CP4 EPSPS expression in tassel tissues.

Example 4

Glyphosate tolerance of transgenic corn plants

R0 Plants:

Transgenic Hi-II corn plants containing pMON25260 were created by particle gun bombardment, transgenic cells selected on glyphosate, and plants were regenerated as described. Transgenic plants were confirmed to contain the CP4 gene by the PCR assay described above. Approximately 5 plants of each individual R0 callus were regenerated and transferred into soil pots and maintained in a greenhouse. Three plants in each line were sprayed with up to 32 oz/acre (2.23 kg/ha) of Roundup® at the time when the fifth leaf emerged. The remaining 2 plants served as the unsprayed controls.

As shown in Table 3, a vegetative score (% growth reduction) of 0-100 was used relative to the unsprayed R0 plants of the same line. A score of 100 was given to plants killed by the Roundup spray, while a 0 represents no visible difference between the sprayed and unsprayed plants. Percent leaf malformation was also used as a measure of vegetative glyphosate tolerance. Flowering and male fertility was evaluated using a rating system of 0-5. A score of 0 was given if no tassel was present, a score of 1 for plants with tassels

which did not undergo anthesis, and score of 5 was given if the plant was fully fertile. The unsprayed plants were rated under the same scoring system.

Lines were selected by RT-PCR for expression of the antisense CP4 gene in the tassels. After spraying with glyphosate at a rate of 32 oz/acre (2.23 kg/ha; line 1 was sprayed at 8 oz/acre (0.56 kg/ha)), 8 transgenic lines showed good to excellent vegetative tolerance (low % growth reduction score) and a high level of male sterility (flowering-male fertility score of 1). Comparing the flowering-male fertility score of the sprayed plants to the unsprayed plants, 7 lines were selected for further characterization. These were lines 1, 2, 3, 6, 11, 13, and 14.

Table 3. Greenhouse evaluation of Ro corn lines for vegetative resistance to glyphosate and fertility scores after spray.

pMON19653/pMON25260
Ro Sprayed with 32 oz/ac (2.23 kg/ha) at the 5-6 leaf stage

Line #	% growth reduction	% leaf malformation	Flowering - male Fertility (sprayed/unsprayed)
1	0	0	2/3.5
2	0	0	1/2.5
3	0	0	1/5.0
4	100	-	-
5			
6	20	5	1/5.0
7	100	-	-
8	100	5	-
9	75	0	1.0
10	100	-	-
11	0	0	1/5.0
12	60	10	nd/1.0
13	0	5	2
14	10	5	1/1.0
15	100	-	-
16	0	0	1/5.0
17	100	-	-

Both sprayed and unsprayed transgenic plants from the selected lines were crossed with pollen from a nontransgenic corn line (B73) to produce F1 seed. Ears produced on plants sterilized by the spray were similar in quality and seed set to that borne on unsprayed

plants, indicating that the plants were fully female fertile under the glyphosate spray conditions used.

Analysis of F1 progeny

F1 progeny from three of the glyphosate-induced male sterile lines were germinated in small soil pots and lines inheriting the transgene were determined by PCR of seedling leaf discs as described above. Vegetative and reproductive tolerance to glyphosate was evaluated by spraying the PCR positive F1 plants with 32 oz/acre (2.23 kg/ha) of Roundup® when they reached the 5-leaf stage. The vegetative and reproductive tolerance was scored as described above. The results are shown in Table 4.

Table 4. Greenhouse evaluation of R1 progeny of glyphosate-induced male sterile corn lines.

F1 data 32 oz/acre (2.23 kg/ha) spray (Line 1 sprayed with 8 oz/acre (0.56 kg/ha)) at 5-6 leaf stage, RT PCR positive F1 progeny

Progeny of Line #	% growth reduction	% malformation	Flowering - male fertility
1 (5910)	0 (8 oz/acre)	0	2
1 (5911)	0 (8 oz/acre)	0	2
1 (5912)	0 (8 oz/acre)	0	3.5
2 (6038)	0	0	1

Each of the three lines showed excellent vegetative scores (growth reduction of 0-10). All three lines showed fertility scores of 1 (complete sterility). It was observed that the anthers from sprayed plants were present, but tightly closed compared to anthers from unsprayed controls that were open and dehiscing copious amounts of pollen. Anthers from sprayed plants were dissected and pollen grains were examined under a dissecting scope. The male sterile anthers produced significantly fewer pollen grains than the fertile controls and the pollen grains that were present were shrunken and abnormal.

Nontransgenic B73 corn pollen was used to pollinate these and other lines to produce seed for field trial evaluation.

Evaluation of lines under field conditions

Table 5 shows the field test results of glyphosate treatment on transgenic corn lines selected from the greenhouse screen for vegetative glyphosate tolerance and male sterility induced by P-Ztap-directed expression of an antisense RNA to the CP4 EPSPS glyphosate tolerance gene. Lines for the field trial were selected based on previous scores of vegetative tolerance, male sterility induced by glyphosate, gene expression, and available seed supply. The pedigree of plants in the field test was either [(Ro x B73) x B73] or (B73 x Ro). Based on these pedigrees, it was expected that half of the plants in a plot would be killed by glyphosate (Roundup®) application since they would not contain the transgene.

The field was set up in two blocks; each block contained four groups of plots intended to receive a single spray treatment. The treatments were 1) No spray, 2) 32 oz/acre (2.23 kg/ha) Roundup® at the 1 2 leaf stage, 3) 32 oz/acre (2.23 kg/ha) Roundup at the 4-5 leaf stage, 4) 32 oz/acre (2.23 kg/ha) Roundup® at the 6-8 leaf stage. Controls included two lines (pMON19653, CP4 EPSPS only) which had previously shown excellent vegetative tolerance to Roundup® but poor reproductive tolerance.

Plants were scored for male fertility when they had begun to flower, and scoring was repeated every 1-2 days for about 8 days. Observations included the dates when the first and last plants in a plot had anthers emerged as well as viability of pollen in those anthers (judged using a hand-held microscope).

The Roundup® sprays at the 6-8 leaf stage were most effective for producing male sterility. Four lines survived the spray and produced tassels. However, in these four lines, no anthers were visible on the 6-8 leaf sprayed plants by the time all plants in the respective unsprayed plots had begun to flower. Upon observation approximately 10 days after the control plots had finished shedding, some anthers were found exserted on the plants which earlier had none. However, in all but one event this number was significantly reduced from normal (e.g. one line had 5 or fewer anthers per plant) and it is expected that such late and abnormally placed anthers would not contain fertile pollen. Plants which appeared to be male-sterile from the Roundup® treatment were pollinated with B73 pollen. As before, seed set from ears borne on treated plants was normal indicating full female fertility.

Table 5. Field evaluation of fertility of transgenic corn plants sprayed with glyphosate (Roundup®) at the 6-8 leaf stage at a rate of 32 oz/acre (2.23 kg/ha).

Line #	Ave. plant height	% anthers exserted[1]	Fertility rating[2]
3	-46%	0	2.0
6	-14%	0	2.5
13	-8%	0	3.0
16	-12%	0	2.0

[1] Percentage of anthers exserted on sprayed plants at the time the unsprayed plants of the same line were shedding pollen

[2] Fertility scoring on sprayed plants 10 days after unsprayed plants were shedding pollen. Scoring system: 0 is completely sterile, no anther formation, 1 is some anther formation, 2 is some anther formation and some pollen shed, 3 is anther formation and some pollen shed, 4 is anther formation and pollen shed but less than fully fertile, 5 is fully fertile. The fertility score on all unsprayed plants was 5.

Example 5

CP4 expression in F1 plants:

F1 progeny from a cross of nontransgenic B73 pollen and the male sterile Hi-II Ro plants were evaluated to determine the CP4 EPSPS expression in leaf and anther tissues using CP4 ELISA assays (20 mg leaf and anther tissues were collected and quick frozen in liquid nitrogen). Total proteins were extracted and quantitated using Bio-Rad protein assay (Bio-Rad Laboratories, Cat#500-0006). CP4 EPSPS protein in leaf and anther tissue extracts was quantitated using *E. coli*-expressed CP4 EPSPS for standard curve. The leaf and anther protein extract from wild type plants were added in standard curve for leaf and anther CP4 EPSPS assay, respectively. CP4 EPSPS levels in all test plants were much higher in leaf tissues than in anther tissues. The ratio of leaf/anther protein expression of CP4 EPSPS in ng CP4 EPSPS/μg soluble protein was determined for corn lines #1, 3 and 6. The range of inhibition of CP4 EPSPS expression was 1.76 X to 14.87 X with the average inhibition of CP4 EPSPS in the anther tissue compared to the leaf tissue being 7.34 X.

Example 6

Utilization of alternative antisense constructs to make Roundup® Ready F1 plants.

Plants are produced using constructs such as described in Example 1 (eg. pMON25235, pMON23232, pMON23233) substituting the constitutive promoter with an anther or pollen-specific promoter in combination with a constitutive promoter providing vegetative and female tolerance to glyphosate. Transgenic lines are chosen that show good vegetative and female tolerance, but male reproductive sensitivity to glyphosate. This trait was backcrossed into inbred lines to be used as females in hybrid crosses.

Fully glyphosate tolerant (Roundup Ready®) corn lines are used as males. These lines contain non-homologous glyphosate tolerance genes such as the plant EPSPS gene or contain different chloroplast targeting peptide and/or intron sequences. Thus, the antisense constructs used to make the female plants male sterile are not active on the glyphosate tolerance genes brought in from the male, and the resulting F1 seeds produce plants which are fully fertile when sprayed with glyphosate.

Example 7

Extension of flower life

Enhancing the length of flower life is an area of significance for the horticultural industry. Petunia species are generally self-incompatible for fertilization. This has allowed for greenhouse observations of the flower life in Petunia species. Petunia varieties V26 and Mitchell have shown that the unpollinated flowers after opening will remain on the plant for 4-6 days with an average of 5 days. Flowers which were cross pollinated by hand at flower opening have a range of flower life of 1.5-3 days with an average of 2.25 days.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the DNA molecules and in the steps or in the sequence of steps of the methods described herein without departing from the concept and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications

apparent to those skilled in the art are deemed to be within the scope and concept of the invention as defined by the appended claims.

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WHAT IS CLAIMED IS:

1. A method for producing male sterile plants comprising the steps of:
inserting into the genome of a plant cell:
a first DNA molecule comprising operably linked in the 5' to 3' orientation:
a first promoter that functions in plant cells to cause the production of a first RNA sequence;
a first DNA sequence encoding a first RNA sequence which encodes a protein that causes tolerance to glyphosate;
a first 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the first RNA sequence; and
a second DNA molecule comprising operably linked in the 5' to 3' orientation:
a second promoter that functions in plant cells to cause the production of a second RNA sequence in a male reproductive tissue;
a second DNA sequence encoding a second RNA sequence that is complementary to the first RNA sequence;
a second 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the second RNA sequence;
obtaining transformed plant cells comprising the first and second DNA molecules;
regenerating transformed plants from the transformed plant cells; and
exposing the transformed plants to glyphosate.

2. The method of claim 1, wherein the first promoter is selected from the group consisting of cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, figwort mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter, and octopine synthase promoter.

3. The method of claim 1, wherein the first DNA sequence encodes a native EPSPS enzyme, a mutant EPSPS enzyme, or a glyphosate degrading enzyme.

4. The method of claim 1, wherein the second promoter is selected from the group consisting of the TA29 tobacco tapetum-specific promoter, PA1 chalcone flavonone isomerase promoter, PA2 chalcone flavonone isomerase promoter, SLG promoter, LAT promoter, exopolygalacuronase promoter, Zmc13 promoter, LAT52 promoter, LAT59 promoter, and psgB6-1 promoter.

5. The method of claim 1, wherein the second RNA sequence is complementary to the protein coding region of the first RNA sequence.

6. The method of claim 1, wherein the second RNA sequence is complementary to a non-translated region of the first RNA sequence.

7. The method of claim 1, wherein the plant is selected from the group consisting of corn, wheat, rice, canola, oat, barley, alfalfa, carrot, cotton, oilseed rape, sugarbeet, sunflower, soybean, tomato, cucumber, and squash.

8. The method of claim 1, further comprising cross-fertilizing of the male sterile plants with pollen from a male fertile donor.

9. The method of claim 8, further comprising harvesting seed from the progeny of the cross-fertilization.

10. A male sterile plant produced by the method of claim 1.

11. A plant cell which contains in its genome:
a first DNA molecule comprising operably linked in the 5' to 3' orientation:
a first promoter that functions in plant cells to cause the production of a first RNA sequence;
a first DNA sequence encoding a first RNA sequence which encodes a protein that causes tolerance to glyphosate;

a first 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the first RNA sequence; and

a second DNA molecule comprising operably linked in the 5' to 3' orientation:

a second promoter that functions in plant cells to cause the production of a second RNA sequence in a male reproductive tissue;

a second DNA sequence encoding a second RNA sequence that is complementary to the first RNA sequence;

a second 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the second RNA sequence.

12. The plant cell of claim 11, wherein the first promoter is selected from the group consisting of cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter, and octopine synthase promoter.

13. The plant cell of claim 11, wherein the first DNA sequence encodes a native EPSPS enzyme, a mutant EPSPS enzyme, or a glyphosate degrading enzyme.

14. The plant cell of claim 11, wherein the second promoter is selected from the group consisting of the TA29 tobacco tapetum-specific promoter, PA1 chalcone flavonone isomerase promoter, PA2 chalcone flavonone isomerase promoter, SLG promoter, LAT promoter, exopolygalacuronase promoter, Zmc13 promoter, LAT52 promoter, LAT59 promoter, and psgB6-1 promoter.

15. The plant cell of claim 11, wherein the second RNA sequence is complementary to a protein coding region of the first RNA sequence.

16. The plant cell of claim 11, wherein the second RNA sequence is complementary to a non-translated region of the first RNA sequence.

17. The plant cell of claim 11, wherein the plant is selected from the group consisting of corn, wheat, rice, canola, oat, barley, alfalfa, carrot, cotton, oilseed rape, sugarbeet, sunflower, soybean, tomato, cucumber, and squash.

18. A plant comprising plant cells of claim 11.

19. Seed comprising plant cells of claim 11.

20. A method of producing hybrid seed comprising the steps of:

producing a male sterile plant by inserting into the genome of a plant cell:

a first DNA molecule comprising operably linked in the 5' to 3' orientation:

a first promoter that functions in plant cells to cause the production of a first RNA sequence;

a first DNA sequence encoding a first RNA sequence which encodes a protein that causes tolerance to glyphosate;

a first 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the first RNA sequence; and

a second DNA molecule comprising operably linked in the 5' to 3' orientation:

a second promoter that functions in plant cells to cause the production of a second RNA sequence in a male reproductive tissue;

a second DNA sequence encoding a second RNA sequence that is complementary to the first RNA sequence;

a second 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the second RNA sequence;

obtaining transformed plant cells comprising the first and second DNA molecules;

regenerating a transformed plant from the transformed plant cells;

increasing the number of transformed plants by growing the transformed plants in the absence of glyphosate;

permitting self-fertilization; and

growing seed from said transformed plants over a number of generations in the absence of glyphosate;

exposing the transformed plants to glyphosate to produce male sterile plants;

effecting cross-fertilization of the male sterile plants with pollen from a male fertile donor, wherein the donor contains in its genome a third DNA molecule comprising operably linked in the 5' to 3' orientation:

a third promoter that functions in plant cells to cause the production of a third RNA sequence;

a third DNA sequence encoding a third RNA sequence that encodes a protein that causes tolerance to glyphosate;

a third 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the third RNA sequence;

wherein the third DNA molecule is different than the first DNA molecule; and

harvesting seed from the progeny of said cross fertilization.

21. The method of claim 20, wherein the first promoter is selected from the group consisting of cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, figwort mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter, and octopine synthase promoter.

22. The method of claim 20, wherein the first DNA sequence encodes a native EPSPS enzyme, a mutant EPSPS enzyme, or a glyphosate degrading enzyme.

23. The method of claim 20, wherein the second promoter is selected from the group consisting of the TA29 tobacco tapetum-specific promoter, PA1 chalcone flavonone isomerase promoter, PA2 chalcone flavonone isomerase promoter, SLG promoter, LAT promoter, exopolygalacuronase promoter, Zmc13 promoter, LAT52 promoter, LAT59 promoter, and psgB6-1 promoter.

24. The method of claim 20, wherein the second RNA sequence is complementary to a protein coding region of the first RNA sequence.

25. The method of claim 20, wherein the second RNA sequence is complementary to a non-translated region of the first RNA sequence.

26. The method of claim 20, wherein the third promoter of the third DNA is selected group consisting of cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, figwort mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter, and octopine synthase promoter.

27. The method of claim 20, wherein the plant is selected from the group consisting of corn, wheat, rice, canola, oat, barley, alfalfa, carrot, cotton, oilseed rape, sugarbeet, sunflower, soybean, tomato, cucumber, and squash.

28. Seed produced by the method of claim 20.

29. A plant produced from the seed of claim 28.

30. A plant cell which contains in its genome

a first DNA molecule comprising operably linked in the 5' to 3' orientation:

a first promoter that functions in plant cells to cause the production of a first RNA sequence;

a first DNA sequence encoding a first RNA sequence which encodes a protein that causes tolerance to glyphosate;

a first 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the first RNA sequence;

a second DNA molecule comprising operably linked in the 5' to 3' orientation:

a second promoter that functions in plant cells to cause the production of a second RNA in a male reproductive tissue;

a second DNA sequence encoding a second RNA sequence that is complementary to the first RNA sequence;

a second 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the second RNA sequence;

a third DNA molecule comprising operably linked in the 5' to 3' orientation:

a third promoter that functions in plant cells to cause the production of a third RNA sequence;

a third DNA sequence encoding a third RNA sequence which encodes a protein that causes tolerance to glyphosate;

a third 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the third RNA sequence;

wherein the third DNA molecule is different than the first DNA molecule.

31. The plant cell of claim 30, wherein the first promoter is selected from the group consisting of cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, figwort mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter, and octopine synthase promoter.

32. The plant cell of claim 30, wherein the first DNA sequence encodes a native EPSPS enzyme, a mutant EPSPS enzyme, or a glyphosate degrading enzyme.

33. The plant cell of claim 30, wherein the second promoter is selected from the group consisting of the TA29 tobacco tapetum-specific promoter, PA1 chalcone flavonone isomerase promoter, PA2 chalcone flavonone isomerase promoter, SLG promoter, LAT promoter, exopolygalacuronase promoter, Zmc13 promoter, LAT52 promoter, LAT59 promoter, and psgB6-1 promoter.

34. The plant cell of claim 30, wherein the second RNA sequence is complementary to a protein coding region of the first RNA sequence.

35. The plant cell of claim 30, wherein the second RNA sequence is complementary to a non-translated region of the first RNA sequence.

36. The plant cell of claim 30, wherein the third promoter is selected from the group consisting of cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, figwort mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter, and octopine synthase promoter.

37. The plant cell of claim 30, wherein the plant is selected from the group consisting of corn, wheat, rice, canola, oat, barley, alfalfa, carrot, cotton, oilseed rape, sugarbeet, sunflower, soybean, tomato, cucumber, and squash.

38. A transgenic plant comprising the plant cells of claim 30.

39. Seed comprising the plant cells of claim 30.

1	AAGCTTATCG	ATACCGTCGA	CCTGCAGGTC	AACGGATCAA	AGGACGCCGC
51	CGAAGTCACC	CGATCGGAGG	ATTGGTCGTC	GATGGACGCC	AGAGCCACCC
101	ACGCCTCTCG	TGGCCGCCAT	CGCCAGAGAC	AACCGCGCCT	CACGCGACCG
151	CCGGAGCGAC	CCGTGTACCT	CGTGGCCACC	GCCGCCGCTG	CCGACGCCTA
201	CCGCGCCTCG	CACAACCGCC	GTTGCTACCG	GGCCTAGGGT	GCGCCACCGC
251	CGCCCGTCCG	CTTCGAGAGC	ACGTTCACCG	CTTGCCCGCC	CGCCTCGGGA
301	GCGTCGCCGT	CGCTCGTTCC	TTGTAGAGCG	CCTGTGATAT	CTTGGCCCCT
351	GGGATGGTGA	TGTCCTGGCC	CAAGGCTTAA	TAGAATTAAT	AAAGTATCTA
401	TACCAATAAG	GTGCATTTTG	TTTTTCGGAA	GCCTATCTCG	AAAGAACCTC
451	CAAGTTAAGT	GTGCTTGGCT	TGGAGCAATT	TTGGATGGGT	GACCGTCCGG
501	GAAGTTTTTC	TCGGGTGCGC	ATGAGTAAGG	ACAAAGTGTT	CACAAAAGAC
551	ATGTGTTGGC	CTGTGGGGAC	AATATATGAT	CCTAGAGAGA	TGCCAGGAGT
601	AAGTACCGCC	GGTCCAGGGA	TTGGACGGGG	TGTTACAAGT	GGTATCAGAG
651	CTGGCCCTCG	CGGTTTCACG	GGTGTGTGTG	GGTTAGGGGT	TCGGGTATAT
701	GGTGCATGTG	GGCCCGAAGT	GGTCACATGG	CATGGTAGGG	GTTCGGGTAT
751	ATGGCGCATG	GCGCATGTGG	GCCCGAAGTG	GTCACATGGT	ATGGTATATG
801	ACGACACTAG	ACACAGACAT	GGCTAAGATG	GGAGGTTCCT	GGATTGGGGT
851	TGACCGACGA	GGACGTCGGT	CTTCTAAGGG	GGGTGGATTG	TGATATCCTG
901	ACCCTTGGGA	TGGTGATGTA	CTGGCCCAAG	GCTTAATAGA	ATTAATAGAG
951	TATCCATACT	AACACGGTGC	ATCTTCTTTT	TCGGAAGCCT	ATCTCGAAAG
1001	AACCTCCAAG	TTAAGTGTGC	TTGGCTTGCA	GTAATCTGGG	ATGGGTGACC
1051	GACCGGGAAG	TTTTTCTCGG	GTGCGCATGA	GTGAGGACAA	AGTGTGCACA
1101	AAAAACCCGT	GTTGGTTTGT	GGGGACAATA	TATGATCCTA	TAGAGCTGCC
1151	AGGAGTAAGT	ACCGCCGGTC	CAGGGATTGG	ACGGGGTGTT	ACAGCGCCGC
1201	CGTCGGTCGC	TCGCCTCGGG	GCGGCACTGC	CGCCGACCCC	TGGATCGCCT
1251	CCACTCGCAC	GCCCGAGGTA	ACCGCCGCCT	CCACTAAAAG	CTAATCCCTG
1301	GCGGTGTGGG	GTTGTTTTTA	TAGCAGCCCG	GACCTGGTCC	AGCTCAACCG
1351	GATTGCACCG	TCTGACATGG	GCTTCCTCTA	GTTATTGGAA	GCCCAGGCCT
1401	AATAATATAC	AATATATATA	TATATATATA	TATATATATA	TATATATATA
1451	TATATATATA	TATATATAAC	AAGTAGACCA	AAAAATGTGA	TATAAAATTT
1501	AAGTGTTTAA	GTGGGACAGG	TGCAACCTTT	TAGTCTATGA	TTTGCCAGAA
1551	ATGATGTAAT	TTTAGTAAAA	CCATTACATT	CTTTTTTTAC	AAATTACTTT
1601	CAGGGCCATC	TATTAGATGC	AATCTAGTTC	CCACTTAAAA	AGAGTTCCAT
1651	TAGTGCAACG	AACCAAATTT	GAAGCACACA	TGGCGAGTGA	TGTATACTCT
1701	AGCTAATAGA	GATTTTTTTT	GTACCAACAA	TGTGTGATTA	ATAATCATAG
1751	AGTTCGGTTT	GCTACTAGTC	ATACGGTCTC	ACACTTTCCT	TACTTTTAAT
1801	CAACTCTTTT	TTATCAGCCG	GGCACTGAAA	CTATGTAGTA	ATACTTGGCT
1851	CACTGCAAAT	TTCTTATAGA	AGCTGGAATA	AAATCCTTTT	AAGGGCTAGT
1901	TTGGGAACCA	CATTTTTCCA	AGGGATTTCA	ATTTTCGCAA	GGGAAATTAG
1951	TTCATTTTCC	CTTGGGAAAA	TAGAAATCCC	ATGGGAAAAT	GTGGTTCCCA
2001	AACTAGCCCT	AAATAAAAAA	AATGAGCAGA	GGAACAGTTC	ACTAGATATG

FIG. 1A

```
2101  TGGCATGAAC  AGAGTAATCC  GGGACGCGCC  ATCAGTGTGG  GTGTGTCATC
2151  CGTGGGAGAC  GCGGGTGCGG  CGCATGAGTC  TGGGATACAG  GGGCCAGTGT
2201  GAGGAGCAGC  TACCATACCA  GGGCACCTAG  TTATTTTTTC  TCAGGGGTGT
2251  TTGGTTACAC  CCCCGCTAAA  ATTTAGCTCC  TATTCCATCG  AATGTTTGAA
2301  CCTCCGTTCC  GGGTATTAAA  TATAGTCGGA  TTATAAAACT  AATTTACCAG
2351  CCGAAGATTA  AAAGACGAGA  CGAATCTAGT  CCAGTTGGTT  GGGTCTATAT
2401  TTCATACTCC  TATTTAAAAG  TCAAACGCTT  GATGTGACCC  GGGCTAAACT
2451  TTAGCAGGAG  CAACCAAACA  CCCGGGTTTG  GAACAAGTTC  ATGAGCAAGG
2501  ACCCTGCACC  GACCACCAAA  GTTCAACGAT  TCACACGCTT  TGGAACTAGA
2551  ACAACTGCTG  TTGGAAACCT  CCTGGTGAAA  TCTCACCCTA  TTAATACCAT
2601  GCTGACGAGC  CAATAGCAGA  AGCATCACAC  ACTAATCAAC  AAGCAGGACC
2651  AGCTAGC
```

FIG. 1B

```
          TCT  AGACTATAAA  ACCACAGCAA  AATGGTAAAG  TATCAATCTT
TATAATCTAA  GTTCAGATTA  CAGAGAAGGA  CGAAACGAAG  TGGGATCC
```

FIG. 2

```
TCT  AGAGGCTGTA  GCCACTGATG  CTGAAATCCT  AAAGGAACAA
AACTTTTGCA  TAAAAATTGA  ATCTTTTTTC  AAAACCAACA  TAGAATTTGC
TGAATTTTTC  AGTTTTTTAG  ATCCAAAAAC  AAGAAAACTT  GAAGATTTAG
GAACTTGGGG  TTTATGGAAA  TTGGAATTGG  GATTAAGGGT  TTGTATCCCT
TGAGCCATGT  TCGGGATCC
```

FIG. 3

```
                TCT  AGAGGCTGTA  GCCACTGATG  CTGAAATCCT
AAAGGAACAA  AACTTTTGCA  TAAAAATTGA  ATCTTTTTTC  AAAACCAACA
TAGAATTTGC  TGAATTTTTC  AGTTTTTTAG  ATCCAAAAAC  AAGAAAACTT
GAAGATTTAG  GAACTTGGGG  TTTATGGAAA  TTGGAATTGG  GATTAAGGGT
TTGTATCCCT  TGAGCCATGT  TGTTAATTTG  TGCCATTCTT  GAAAGATCTC
CACTACAGTT  GTCCCTATAA  AACCACAGCA  AAATGGTAAA  GTATCAATCT
TTATAATCTA  AGTTCAGATT  ACAGAGAAGG  ACGAAACGAA  CTGGGATCC
```

FIG. 4

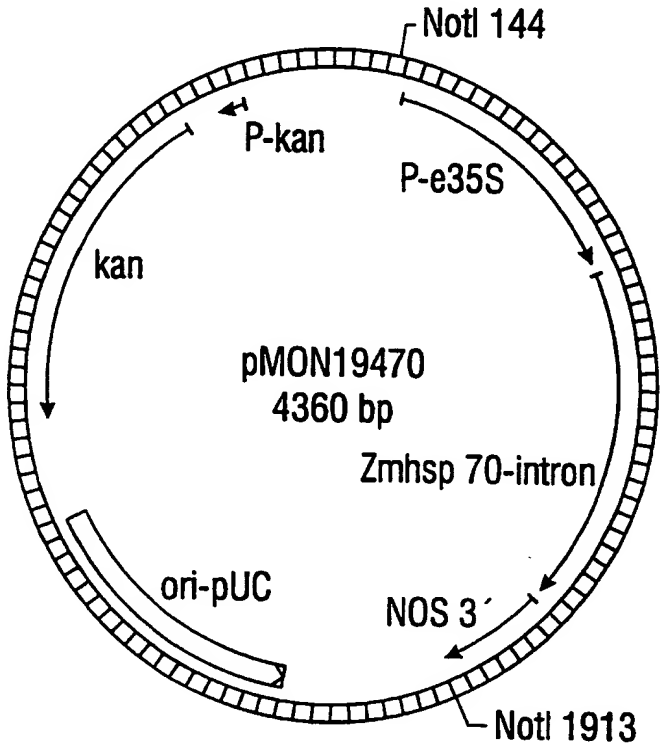
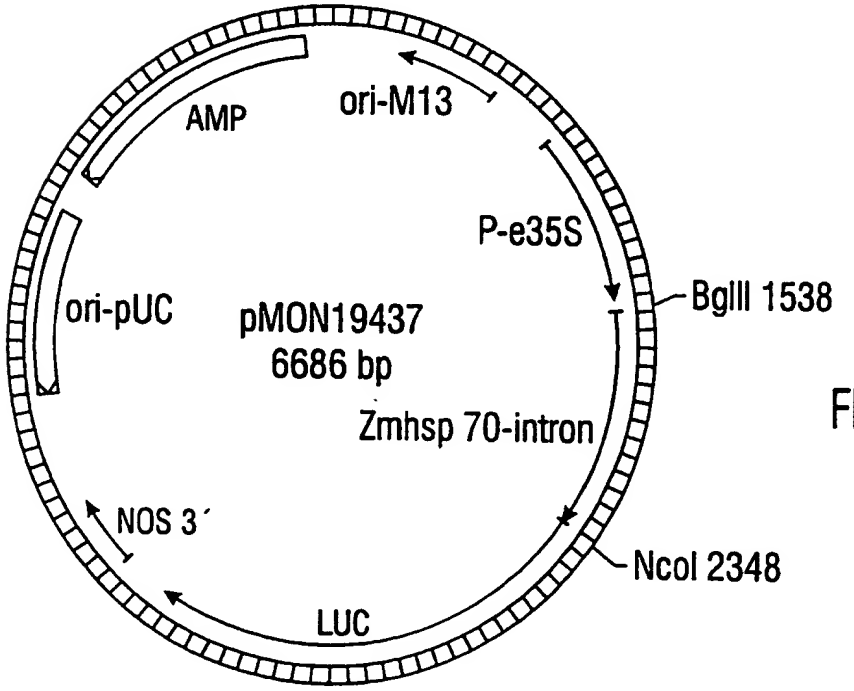

NotI 144
P-kan
P-e35S
kan
pMON19470
4360 bp
Zmhsp 70-intron
ori-pUC
NOS 3´
NotI 1913

FIG. 5A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ori-M13
P-e35S
BglII 1538
ori-pUC
pMON19437
6686 bp
Zmhsp 70-intron
NOS 3´
NcoI 2348
LUC

FIG. 5B

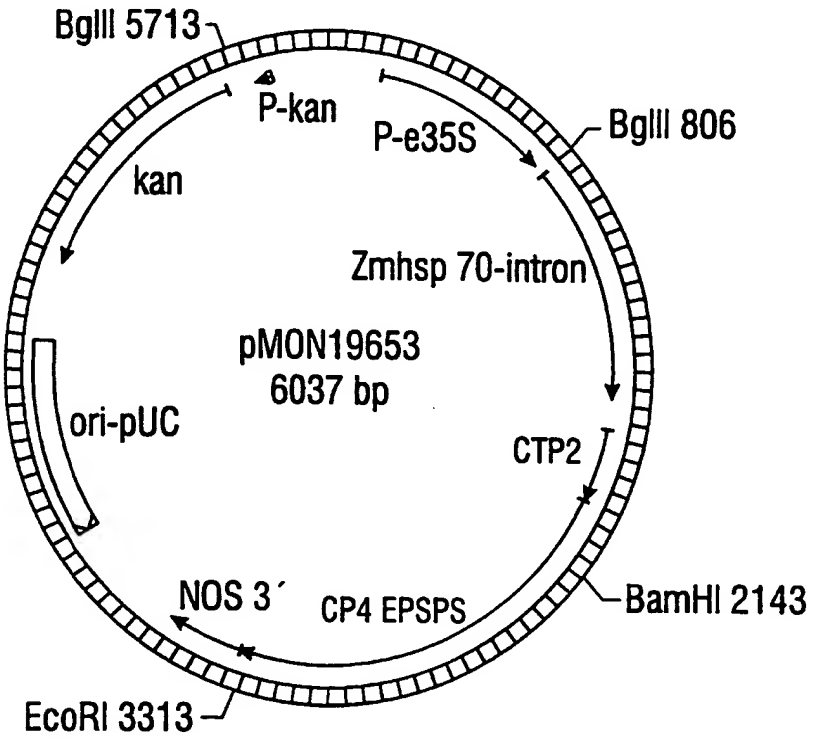
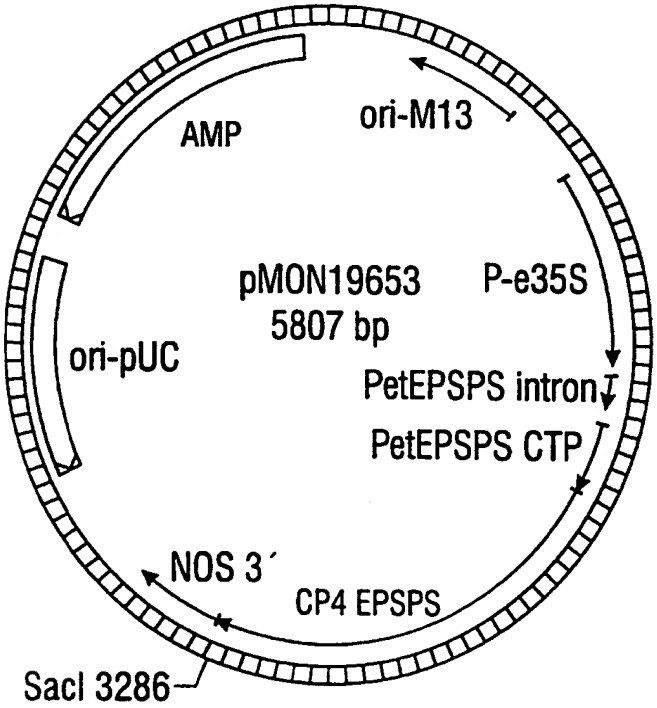

BglII 5713
P-kan
P-e35S
BglII 806
kan
Zmhsp 70-intron
pMON19653
6037 bp
ori-pUC
CTP2
NOS 3´
CP4 EPSPS
BamHI 2143
EcoRI 3313

FIG. 6A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ori-M13
pMON19653
5807 bp
P-e35S
ori-pUC
PetEPSPS intron I
PetEPSPS CTP
NOS 3´
CP4 EPSPS
SacI 3286

FIG. 6B

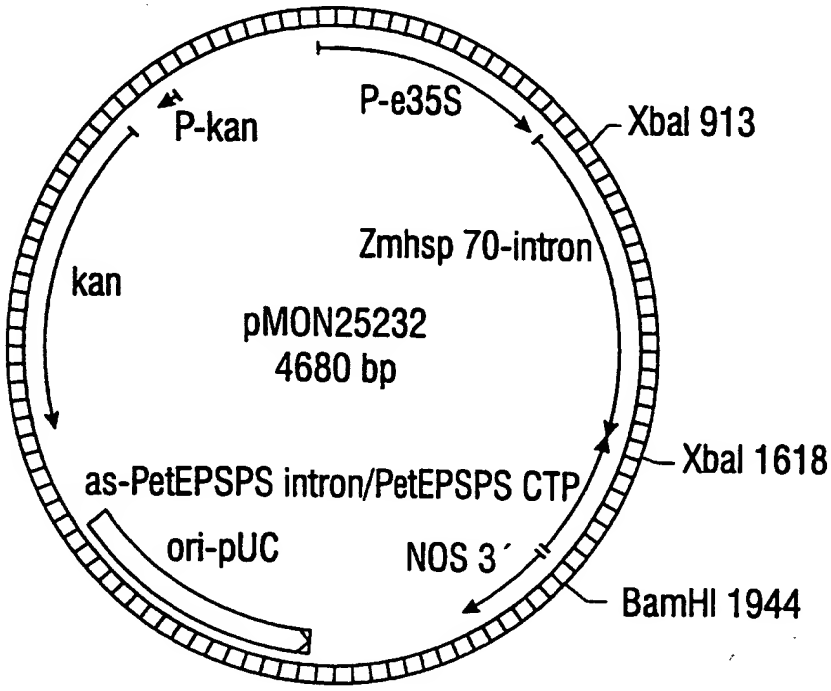
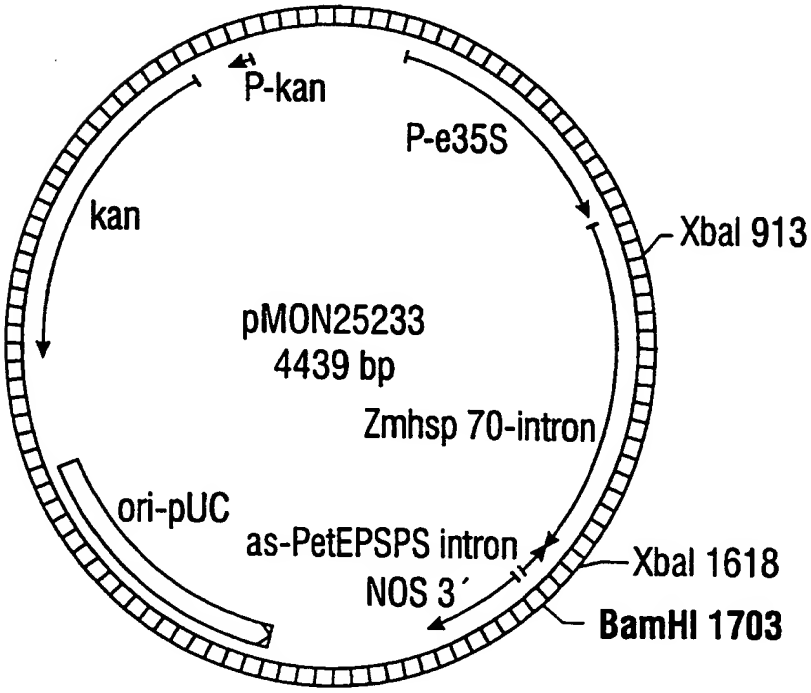

P-kan
P-e35S
XbaI 913
Zmhsp 70-intron
kan
pMON25232
4680 bp
XbaI 1618
as-PetEPSPS intron/PetEPSPS CTP
ori-pUC
NOS 3´
BamHI 1944

FIG. 7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P-e35S
kan
XbaI 913
pMON25233
4439 bp
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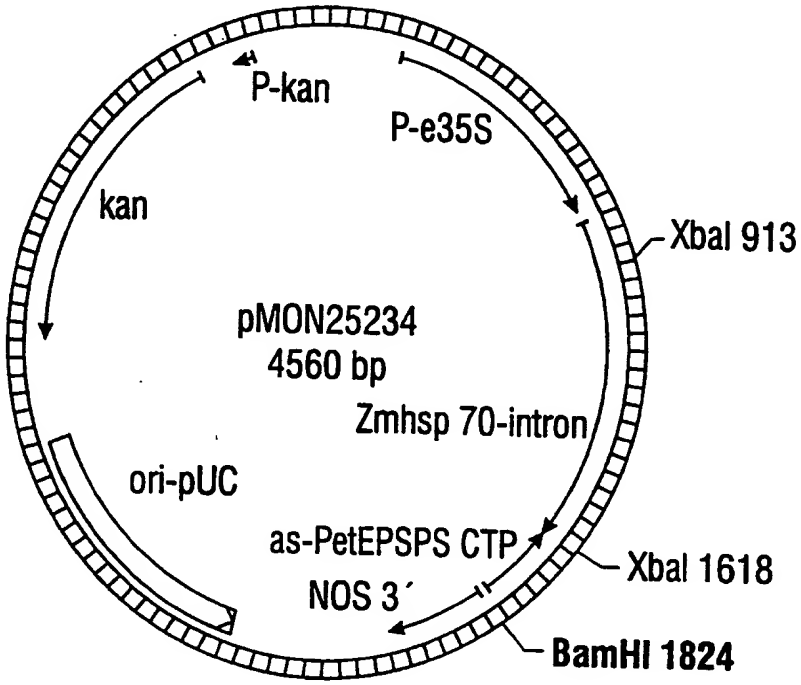
ori-pUC
as-PetEPSPS intron
XbaI 1618
NOS 3´
BamHI 1703

FIG. 8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kan
XbaI 913
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ori-pUC
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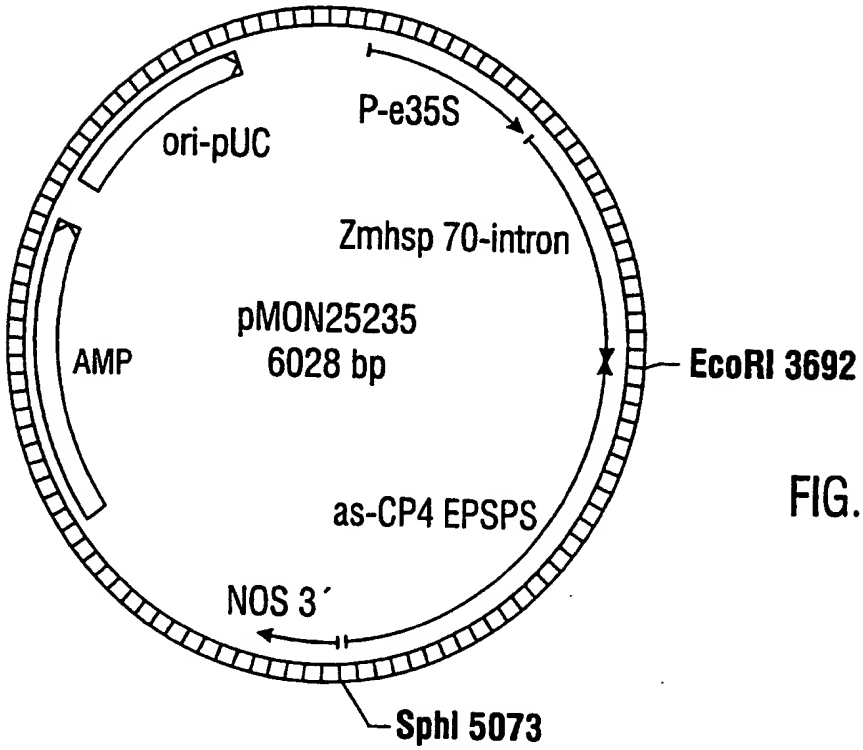
XbaI 1618
NOS 3´
BamHI 1824

FIG. 9A


P-e35S
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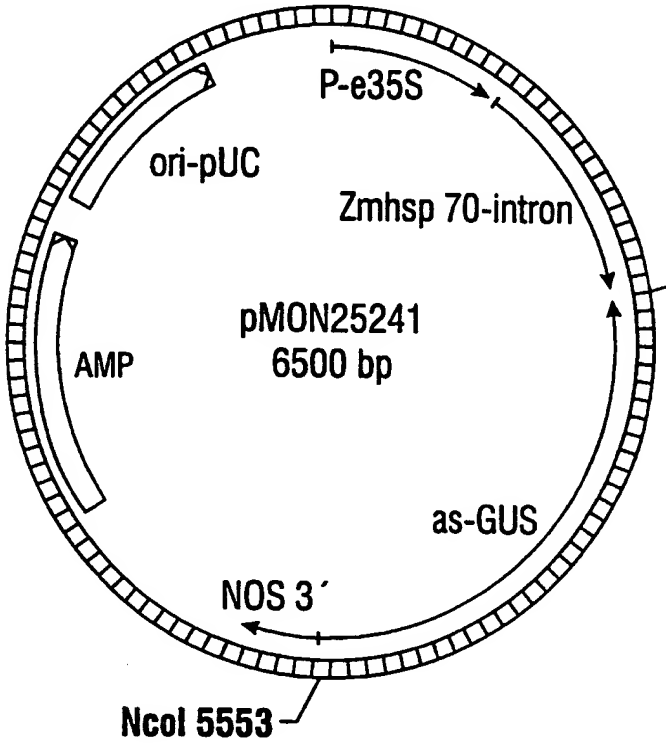
AMP
EcoRI 3692
as-CP4 EPSPS
NOS 3´
SphI 5073

FIG. 9B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EcoRI 3692
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6500 bp
AMP
as-GUS
NOS 3´
NcoI 5553

FIG. 10

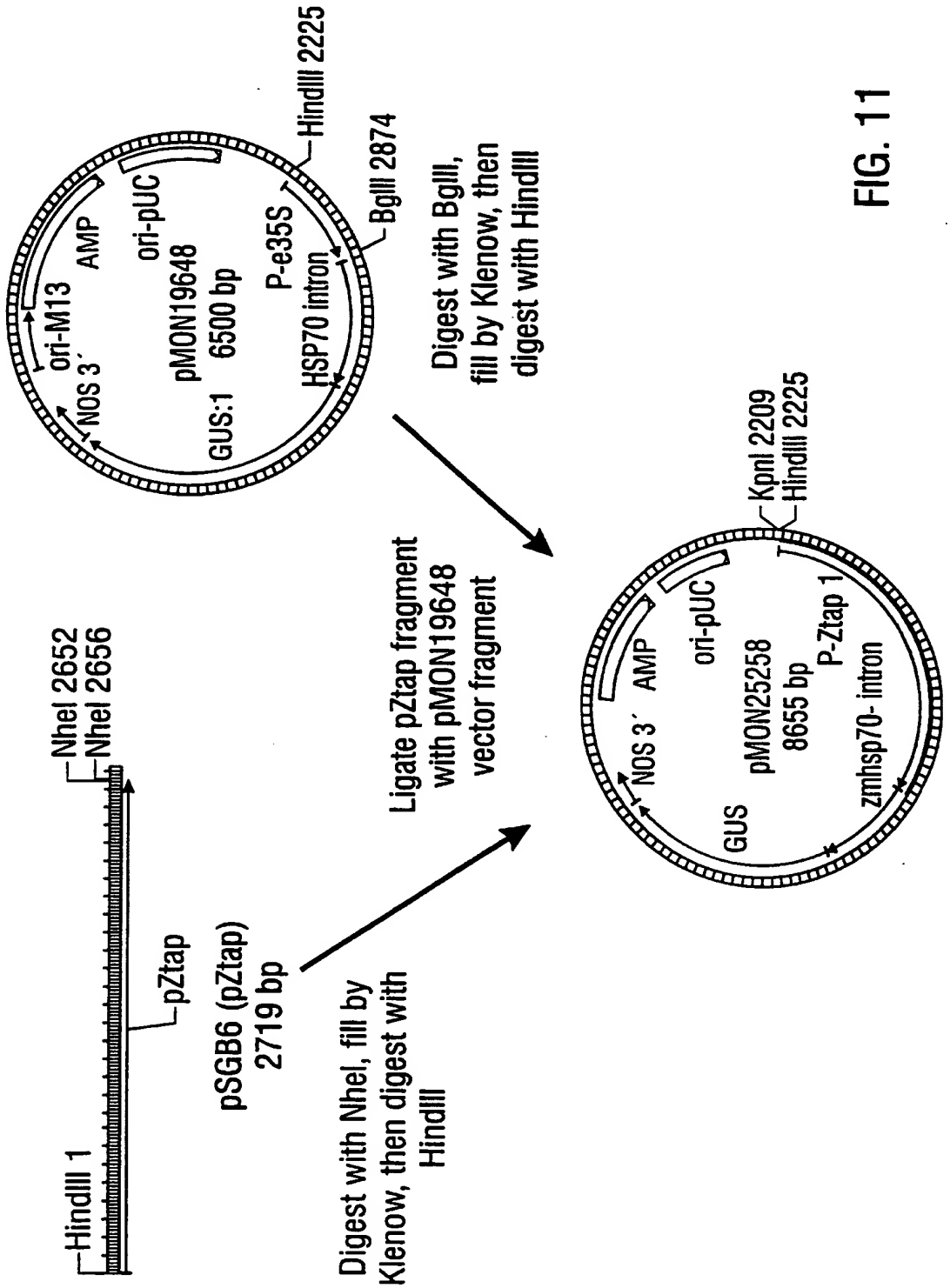

HindIII 2225
BglII 2874
ori-M13
AMP
ori-pUC
NOS 3'
pMON19648
6500 bp
P-e35S
HSP70 intron
GUS:1
Digest with BglII, fill by Klenow, then digest with HindIII
NheI 2652
NheI 2656
pZtap
pSGB6 (pZtap)
2719 bp
HindIII 1
Digest with NheI, fill by Klenow, then digest with HindIII
Ligate pZtap fragment with pMON19648 vector fragment
KpnI 2209
HindIII 2225
ori-pUC
AMP
NOS 3'
pMON25258
8655 bp
P-Ztap 1
zmhsp70- intron
GUS

FIG. 11

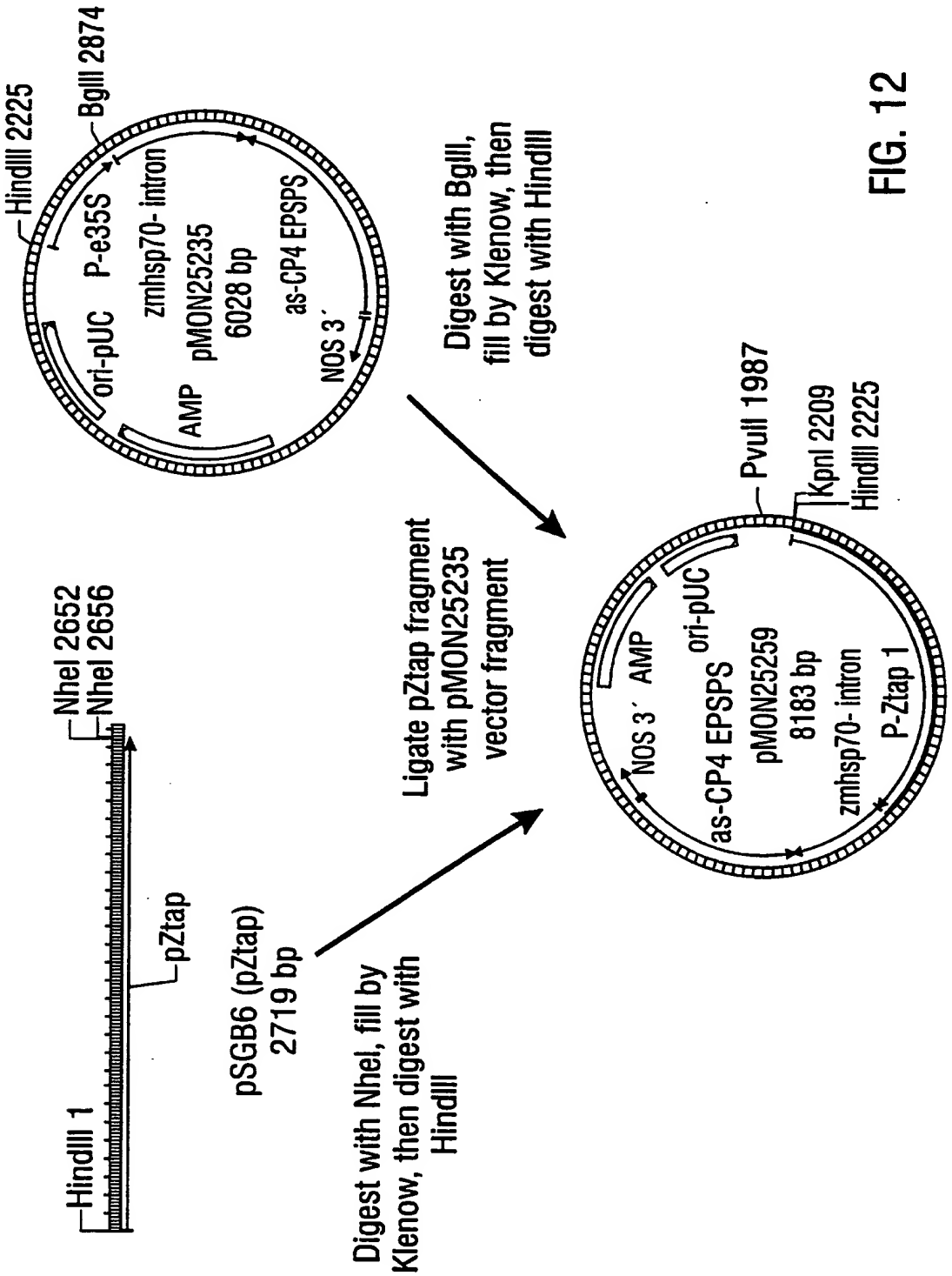
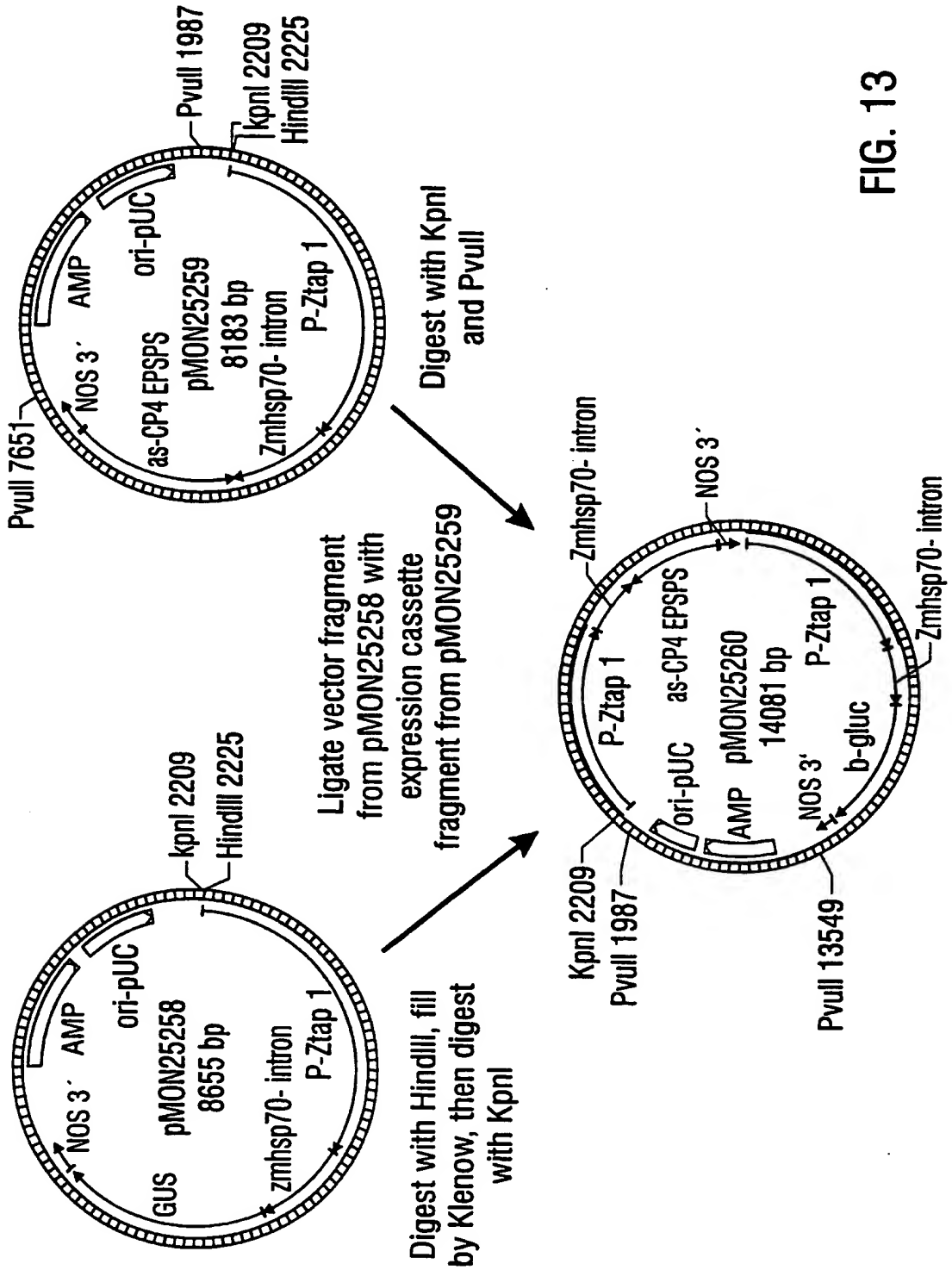

HindIII 2225
BglII 2874
P-e35S
zmhsp70- intron
ori-pUC
pMON25235
6028 bp
AMP
as-CP4 EPSPS
NOS 3´
Digest with BglII, fill by Klenow, then digest with HindIII
PvuII 1987
KpnI 2209
HindIII 2225
NOS 3´ AMP
as-CP4 EPSPS
ori-pUC
pMON25259
8183 bp
zmhsp70- intron
P-Ztap 1
Ligate pZtap fragment with pMON25235 vector fragment
NheI 2652
NheI 2656
pZtap
HindIII 1
pSGB6 (pZtap)
2719 bp
Digest with NheI, fill by Klenow, then digest with HindIII

FIG. 12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NOS 3'
AMP
ori-pUC
as-CP4 EPSPS
pMON25259
8183 bp
Zmhsp70- intron
P-Ztap 1
PvuII 1987
KpnI 2209
HindIII 2225
Digest with KpnI and PvuII
Ligate vector fragment from pMON25258 with expression cassette fragment from pMON25259
Digest with HindIII, fill by Klenow, then digest with KpnI
NOS 3'
AMP
ori-pUC
GUS
pMON25258
8655 bp
zmhsp70- intron
P-Ztap 1
KpnI 2209
HindIII 2225
Zmhsp70- intron
NOS 3'
P-Ztap 1
as-CP4 EPSPS
ori-pUC
AMP
pMON25260
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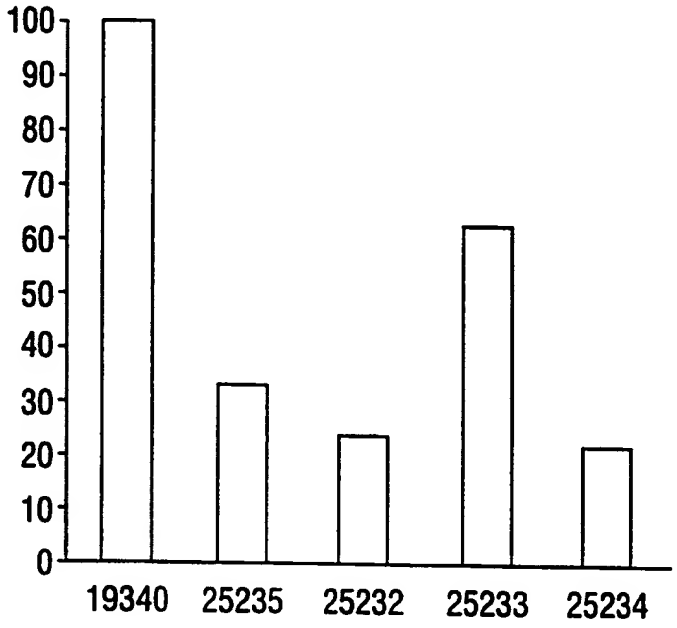
NOS 3'
P-Ztap 1
b-gluc
Zmhsp70- intron
KpnI 2209
PvuII 1987
PvuII 13549

FIG. 13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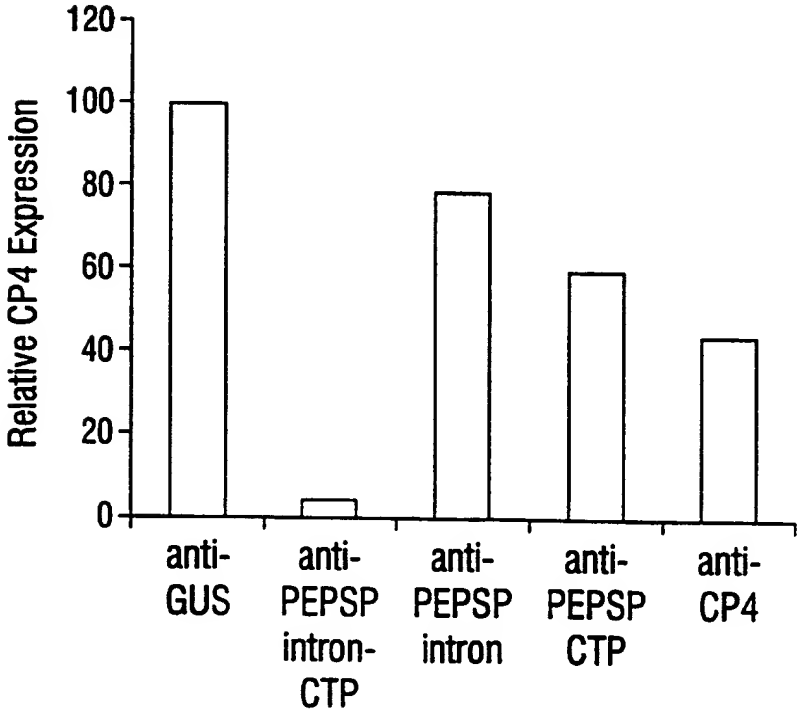
70
60
50
40
30
20
10
0
19340
25235
25232
25233
25234

FIG. 14


Relative CP4 Expression
120
100
80
60
40
20
0
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anti-PEPSP intron-CTP
anti-PEPSP intron
anti-PEPSP CTP
anti-CP4

FIG. 15

SEQUENCE LISTING

<110> BROWN, SHERRI
FROMM, MICHAEL

<120> GLYPHOSATE AS A GAMETOCIDE

<130> MOBT122

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<141>

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<151> 1998-03-09

<160> 9

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gcgtcgccgt cgctcgttcc ttgtagagcg cctgtgatat cttggcccct gggatggtga 360
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gcccgaggta accgccgcct ccactaaaag ctaatccctg gcggtgtggg gttgttttta 1320
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agggatttca attttcgcaa gggaaattag ttcattttcc cttgggaaaa tagaaatccc 1980

atgggaaaat gtggttccca aactagccct aaataaaaaa aatgagcaga ggaacagttc 2040
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